Vitamin D status and latent tuberculosis infection: a preliminary study in a group of healthy Mexican agricultural workers

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ABSTRACT

Vitamin D metabolites are important in the regulation of bone and calcium homeostasis, but also have a more ubiquitous role in the regulation of cell differentiation and immune function. Severely low circulating 25-dihydroxyvitamin D [25(OH)D] concentrations have been associated with the onset of active tuberculosis (TB) in immigrant populations, although the association with latent TB infection (LTBI) has not received much attention. A previous study identified the prevalence of LTBI among a sample of Mexican migrant workers enrolled in Canada's Seasonal Agricultural Workers Program (SAWP) in the Niagara Region of Ontario. The aim of the present study was to determine the vitamin D status of the same sample, and identify if a relationship existed with LTBI.

Studies of vitamin D deficiency and active TB are most commonly carried out among immigrant populations to non-endemic regions, in which reactivation of LTBI has occurred. Currently, there is limited knowledge of the association between vitamin D deficiency and LTBI. Entry into Canada ensured that these individuals did not have active TB, and LTBI status was established previously by an interferon-gamma release assay (IGRA) (QuantiFERON-TB Gold In-Tube®, Cellestis Ltd., Australia). Awareness of vitamin D status may enable individuals at risk of deficiency to improve their nutritional health, and those with LTBI to be aware of this risk factor for disease.

Prevalence of vitamin D insufficiency among the Mexican migrant workers was determined from serum samples collected in the summer of 2007 as part of the cross

sectional LTBI study. Samples were measured for concentrations of the main circulating vitamin D metabolite, 25(OH)D, with a widely used ^{125}I 25OHD RIA (DiaSorin Inc.®, Stillwater, MN), and were categorized as deficient (<37.5 nmol/L), insufficient (>37.5 nmol/L, < 80 nmol/L) or sufficient (≥80 nmol/L). Fisher's exact tests and t tests were used to determine if vitamin D status (sufficiency or insufficiency) or 25(OH)D concentrations significantly differed by sex or age categories. Predictors of vitamin D insufficiency and 25(OH)D concentrations were taken from questionnaires carried out during the previous study, and analyzed in the present study using multiple regression prediction models. Fisher's exact test and t test was used to determine if vitamin D status or 25(OH)D concentration differed by LTBI status. Strength of the relationship between interferon-gamma (IFN-γ) concentration (released by peripheral T cells in response to TB antigens) and 25(OH)D concentration was analyzed using a Spearman correlation.

Out of 87 participants included in the study (78% male; mean age 38 years), 14 were identified as LTBI positive but none had any signs or symptoms of TB reactivation. Only 30% of the participants were vitamin D sufficient, whereas 68% were insufficient and 2% were deficient. Significant independent predictors of lower 25(OH)D concentrations were sex, number of years enrolled in the SAWP and length of stay in Canada. No significant differences were found between 25(OH)D concentrations and LTBI status. There was a significant moderate correlation between IFN-γ and 25(OH)D concentrations of LTBI-positive individuals.

The majority of participants presented with Vitamin D insufficiency but none were severely deficient, indicating that 25(OH)D concentrations do not decrease dramatically

in populations who temporarily reside in Canada but go back to their countries of origin during the Canadian winter. This study did not find a statistical relationship between low levels of vitamin D and LTBI which suggests that in the presence of overall good health, lower than ideal levels of 25(OH)D, may still be exerting a protective immunological effect against LTBI reactivation. The challenge remains to determine a critical 25(OH)D concentration at which reactivation is more likely to occur.

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LIST OF ABBREVIATIONS

$1,25(OH)_2D$: 1α,25-dihydroxyvitamin D

25(OH)D: 25-hydroxyvitamin D

7-DHC: 7-dehydrocholesterol

AI: Adequate intake

APC: Antigen presenting cell

BCG vaccine: Bacille Calmette-Guérin vaccine

BMD: Bone mineral density

CV: Coefficient of variation

DBP: Vitamin D binding protein

DEQAS: Vitamin D External Quality Assessment Scheme

ELISA: Enzyme-linked immunosorbent assay

HPLC: High performance liquid chromatography

IGRA: Interferon-gamma release assay

IFN: Interferon

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LTBI: Latent tuberculosis infection

NO: Nitric oxide

NOS2: Nitric oxide synthase 2

PTH: Parathyroid hormone

QFT: QuantiFERON-TB Gold In-tube

RIA: Radioimmunoassay

SAWP: Seasonal Agricultural Workers Program

TB: Tuberculosis

Th: T helper

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TST: Tuberculin skin test

VDR: Vitamin D receptor

VDRE: Vitamin D response elements

WHO: World Health Organization

CHAPTER ONE: INTRODUCTION

Vitamin D is primarily recognized for its effects on bone health. Although it is referred to as a vitamin by name, its ability to be produced endogenously and the function of its metabolites as intercellular messengers denotes that vitamin D, in fact, acts as a hormone [1]. Recently, the regulatory properties of the hormone have been described for multiple biological processes and its deficiency has been established as a risk factor for several chronic and infectious diseases, particularly tuberculosis (TB) [2].

The role of vitamin D in combating *Mycobacterium tuberculosis*, the causal agent of TB, has been observed and applied since the 1800s. During this time, exposure to sunlight and the administration of cod liver oil (both sources of vitamin D) were observed as having beneficial effects for TB patients in sanatoria, who were said to have regained their strength within days [3, 4]. The biological mechanisms leading to the health improvement of TB patients undergoing these treatments were unknown at the time, but became evident when vitamin D was isolated from cod liver oil in 1922 by Elmer V. McCollum [5]. Oral doses of vitamin D were administered routinely to TB patients until the discovery of TB antibiotics in the 1940s, from which time its use to treat TB began to lessen within the mainstream medical community [2].

After many decades of seemingly ignoring the potential role of vitamin D as supplementary aid in the treatment of TB, increasing evidence now confirms that there is a significant association between decreased vitamin D levels and active TB disease [6]. This is especially true in patients belonging to certain ethnic groups [7, 8], immigrants to

non-endemic countries [9], and others in whom insufficient cutaneous synthesis or dietary intake may lead to hypovitaminosis D [6, 10, 11].

Supporting evidence for the contribution of vitamin D to improved host resistance to TB is derived from the recent elucidation of the mechanism by which the biologically active form of vitamin D, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$), enhances the antituberculous response of macrophages, cells of the innate immune system which are the main targets for *M. tuberculosis* infection.

This newly established link between the hormonally active metabolite and the innate immune system has greatly contributed to the increasing interest in investigating the beneficial role of vitamin D not only in active TB, but in latent infection as well [12]. In latent TB infection (LTBI), mycobacteria evade intracellular killing by inhibiting the fusion of the phagosome and lysosome within the macrophage. It has been shown that vitamin D metabolites can reverse this inhibition by promoting phagolysosomal fusion [13]. Thus, the role of vitamin D in the prevention of LTBI reactivation is worth exploring. If proven beneficial, vitamin D supplementation in individuals with demonstrated LTBI could potentially reduce the global burden of one of the world's most widespread diseases [14]. The present study will investigate vitamin D deficiency as a risk factor for TB reactivation in an otherwise healthy population from a TB-endemic country.

CHAPTER TWO: LITERATURE REVIEW

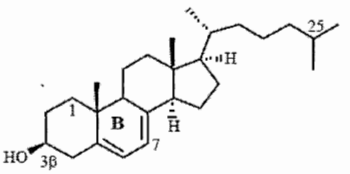
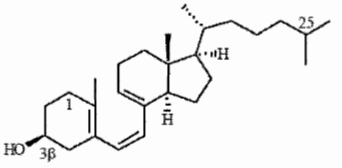
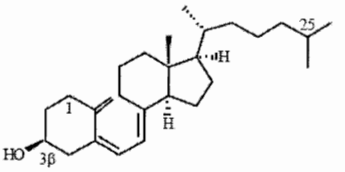
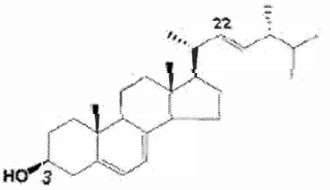
Though commonly referred to as the "sunshine vitamin", vitamin D metabolites act as hormones and have long been acknowledged to be essential for the proper development of bones, especially in the prevention of rickets among children. During 18^{th} and 19^{th} centuries, the Industrial Revolution [15] led a large number of people to relocate from rural to urban homes and jobs. Urban habitation often resulted in increased malnutrition, indoor employment and increased smog exposure that reduced exposure to sunlight. During this time, there was an increase in the incidence of rickets, a softening of the bones due to vitamin D deficiency [16, 17]. From 1918 to 1922, the ability of cod liver oil to reverse the symptoms of rickets was recognized and described by Edward Mellanby and Elmer V. McCollum [18, 19]. The substance providing these protective properties was subsequently isolated and named vitamin D in 1922, by McCollum [5]. Recently, vitamin D has been receiving increased attention for its role in maintaining health and homeostasis in a variety of essential body processes, and in the prevention of numerous chronic and infectious diseases [20, 21]. The protective role of vitamin D against active TB disease extends back to the 1800s, when the benefits of cod liver oil and sunlight were applied as TB therapies [2].

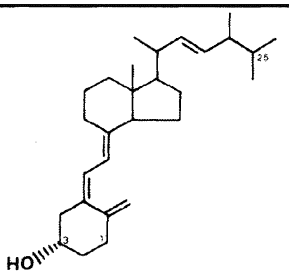
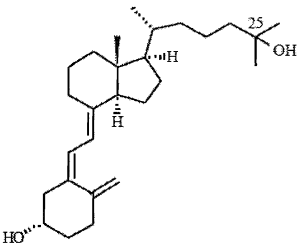
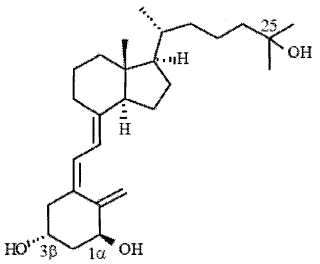
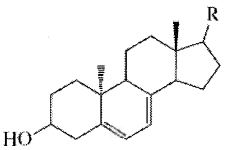
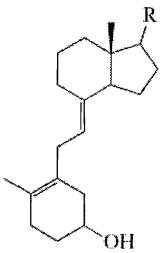
2.1 The Vitamin D Molecule

There are two forms of vitamin D, vitamins D_2 and D_3, which can be utilized by humans. Both can be intestinally absorbed, vitamin D_2 by the consumption of some plants and mushrooms and vitamin D_3 by the consumption of fatty fish and fortified foods [22].

Generally, vitamin D_3 is endogenously produced in the skin in response to ultraviolet B (UVB) radiation. Vitamins D_2 and D_3 are biologically inert molecules, and must go through a series of enzymatically induced alterations, producing multiple metabolites, before biologically active forms are produced [23]. $1,25(OH)_2D$ is the biologically active metabolite, although 25(OH)D is most frequently measured. Table 1 summarizes the main functions of these metabolites. The critical molecular difference from the D_2 forms is in the ring B structure. The remainder of the document will use vitamin D to refer to either D_2 or D_3, unless otherwise specified.

Table 1: The structures and functions of selected vitamin D metabolites and other associated molecules*.

Vitamin D Metabolite	Structure	Functions
7-dehydrocholesterol (or 5,7-cholestradienol)		An inert molecule in the skin that absorbs UVB radiation of 290-315 nm at the two double bonds of ring B.
Pre-vitamin D_3 (or precalciferol)		Thermodynamically unstable molecule that isomerizes within 2-3 days, acting as the mediator between 7-DHC and vitamin D_3.
Vitamin D_3 (or cholecalciferol, calciol)		Produced in the skin by the UVB irradiation of pre-vitamin D_3, and diffuses into the blood, through which it is carried by DBP.
Pre-vitamin D_2 (or ergosterol)		A common steroid produced by plants which is converted to vitamin D_2 following UVB irradiation.

Vitamin D_2 (or ergocalciferol)		Analogue of vitamin D in plants which is derived from the UVB irradiation of pre-vitamin D_2. This is a dietary source of vitamin D.
25-hydroxyvitamin D_3 (or calcidiol)		Produced in the liver by the hydroxylation of vitamin D_3 (pictured) or D_2. It is the most commonly circulating form of vitamin D, making it a good biomarker for vitamin D status, although it is biologically inert.
1,25-dihydroxyvitamin D_3 (or calcitriol)		Produced primarily in the kidneys by the hydroxylation of 25(OH)D_3, it is the hormonally active form of vitamin D.
Lumisterol		Produced by the irradiation of 7-DHC. This molecule is biologically inert and is naturally sloughed off with the skin.
Tachysterol		Synthesized by the further irradiation of pre-vitamin D_3. This molecule is biologically inert and is naturally sloughed off with the skin.

*Adapted from Holick [24]Johnson et al [23] , Peleg et al [25], Fraser et al [26], Kobori et al [27], Orlova et al [28], Caballero et al [29], Gropper et al [30].
DBP, Vitamin D binding protein; UVB, ultra violet blue; 7-DHC, 7-dehydrocholesterol

2.2 Sources of Vitamin D

2.2.1 Dietary Sources

Vitamin D is a unique molecule, as it can be both endogenously synthesized and obtained through diet or supplements. Animals are only able to synthesize vitamin D_3, while plants and mushrooms produce only vitamin D_2. The primary dietary source of vitamin D_3 is the flesh of oily fish. A common steroid produced by plants is ergosterol (or pre-vitamin D_2), which is converted to vitamin D_2 following UVB irradiation [22].

In 1997, the Dietary Reference Intakes for the Institute of Medicine [31] in the United States established the adequate intake (AI) of vitamin D for males and females at different ages, as depicted below in Table 2. The AI values were determined by the levels of dietary vitamin D required to prevent a wintertime decrease in serum 25(OH)D concentrations among adults 19-50 years of age [32].

Table 2: The recommended adequate intake of vitamin D (IU/day) for males and females of various age groups* [31]

Age	Adequate Intake (IU/day)	Adequate Intake (µg/day)
≤50 years (and all pregnant and lactating women)	200	5
51-70 years	400	10
>70 years	600	15

* Adequate intake is converted from IU/day to µg/day by multiplying by 40

The established consensus on the adequate intake (AI) of vitamin D has met with inconsistencies. Despite the above recommendations, the Canadian Paediatric Society recommends an AI of 400 IU/day for infants and the Canadian Osteoporosis Society

recommends an AI of 800 IU/day for adults ≥51 years at risk of osteoporosis [33]. As well, multiple studies have shown that these AI values may not be adequate enough to meet daily vitamin D requirements. For example, a Canadian study [34] of adults (ages ranging 27-89 years) showed that almost everyone taking the recommended 200 IU/day vitamin D supplementation still had insufficient 25(OH)D concentrations (<80 nmol/L) at least one time during the year, indicating that the recommended daily intake levels may not be adequate. Bischoff-Ferrari and colleagues [21] carried out a review of serum 25(OH)D concentration thresholds, concluding that optimal 25(OH)D concentrations begin at 75 nmol/L. This conclusion was based on findings that bone mineral density (BMD) and dental health were improved, while fractures and colorectal cancer were prevented. Further, 25(OH)D concentrations ≥75 nmol/L could not be reached with the current recommended daily intakes of 200-600 IU vitamin D. In another review, Vieth [35] reasoned that at least 800 IU/day are required to promote overall good health. In fact, Lu and colleagues [36] stated that without adequate sun exposure, dietary vitamin D should be at least 1000 IU/day. Since one full body UVB exposure has been shown to yield serum 25(OH)D concentrations equivalent to one vitamin D supplementation dose of 10,000 IU, and current AI recommendations may need reassessment [35]. As well, the absence of toxicity in clinical trials using this vitamin D supplement dose indicates that this may be an appropriate upper intake level of vitamin D [37].

As depicted above in Table 2, AI values differ with respect to age. Studies of the 25(OH)D concentrations of older age groups have shown that recommended levels may be inadequate to maintain vitamin D sufficiency, as indicated in the following two studies

of women in the United States. Firstly, Lappe and colleagues [38] found that women aged 55 years and over taking vitamin D supplementation of 1100 IU/day for four years, were found to have an average increase in serum 25(OH)D concentration of approximately 23.9 nmol/L, raising the serum concentration to greater than 80 nmol/L, considered the level of sufficiency. Secondly, Aloia and colleagues [39] found that serum 25(OH)D concentrations were only increased above 80 nmol/L after supplementation of 2000 IU/day.

Monitoring vitamin D intake through dietary consumption may be problematic at times. As previously stated, vitamin D is absorbed from some naturally occurring foods, such as fatty fish, but some foods, such as milk and margarine, are vitamin D-fortified. Fortification systems may have inconsistencies, resulting in inaccurate estimations of dietary vitamin D intake. For example, a study of Aboriginal and non-Aboriginal Canadian women reported that although each participant was meeting the AI of dietary vitamin D, primarily through the consumption of fortified milk and margarine, vitamin D deficiency (in this study defined as serum 25(OH)D concentration <37.5 nmol/L) was found in all participants [40]. This may have been due, in part, to discrepancies in the fortification system of foods in North America. Despite the fact that Canada and the US fortify milk with approximately 385-425 IU vitamin D per litre of milk [41, 42], 49% of milk samples tested in the US and British Columbia contained less than 80% of the vitamin D content on the label and 14% did not have any detectable levels of vitamin D [43]. This may lead to deficiencies in individuals living at high latitudes, who rely on dietary vitamin D during a portion of the year.

Similarly, the vitamin D_3 content of fish has shown variation. For example, Lu and colleagues [36] have demonstrated that wild-caught salmon contained approximately 988 IU vitamin D_3/100 g serving, while the vitamin D_3 content of farmed salmon was approximately 25% that of wild salmon. As well, it should be noted that while cooking salmon had a negligible effect in this study, frying in vegetable oil reduced vitamin D_3 content by about 50%.

2.2.2 Sun Exposure

Humans are capable of cutaneously producing vitamin D_3 in response to UVB exposure, and this is the most abundant source of vitamin D for most humans [44]. The human sebaceous glands produce 7-dehydrocholesterol (7-DHC), an inert molecule that is secreted and evenly distributed throughout the epidermal and dermal layers of the skin [30]. The two double bonds of ring B of 7-DHC (Table 1) absorb UVB radiation (290-315 nm wavelength) that converts the molecule to pre-vitamin D_3. Within 2-3 days, pre-vitamin D_3 is thermally isomerized into vitamin D_3 and diffuses from the skin into the blood, through which it is transported by the transport α-2 globulin vitamin D-binding protein (DBP) (Figure 1) [24, 45].

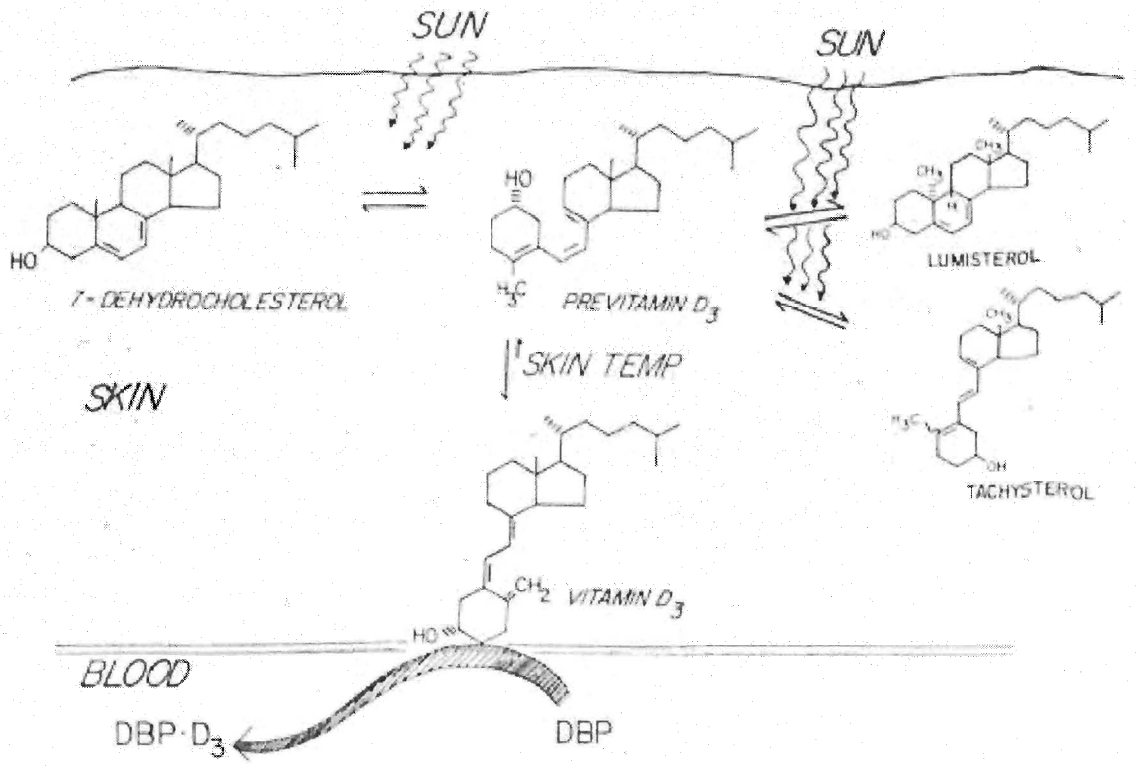


Figure 1: Cutaneous production of pre-vitamin D_3 following UVB exposure [24]

In order to prevent vitamin D toxicity, there are control mechanisms in place. Other molecules produced during UVB irradiation are lumisterol and tachysterol, synthesized by the further irradiation of pre-vitamin D_3 (Figure 1 and Table 1). These compounds are biologically inert and are naturally sloughed off with dead skin cells, thereby preventing the overproduction of vitamin D_3 in the skin resulting from sunlight over-exposure [46]. Also contributing to the prevention of endogenous overproduction, is the degradation of vitamin D_3 in response to excess sunlight exposure [47].

Regardless of diet, the greatest source of vitamin D is through cutaneous production by way of UVB radiation. As previously stated, the wavelength of UVB ranges between

290-315 nm, and its absorption by the skin is dependent on various factors, stemming from the sun's position during different times of the year [24]. The amount of UVB that reaches the biosphere is affected by the zenith angle of solar radiation and the amount of ozone in the atmosphere, which absorbs UVB very efficiently. These factors are dependent on latitude, season, and time of day. The zenith angle changes with the daily rotation of the Earth, and is a measure of the angle at which the sun's rays reach the earth. As the zenith angle reaches 90°, a greater amount of UVB is available for absorption. At higher latitudes, especially during the winter months, the shorter wavelengths of UVB are absorbed and scattered by the atmosphere. This leaves the inhabitants of higher latitudes with reduced exposure, which will in turn result in less synthesis and subsequent absorption of vitamin D in the skin [43, 48]. As well, between October and March, individuals living at latitudes greater than 42° north or south are not exposed to sufficient UVB photons for cutaneous vitamin D_3 production (Figure 2) [49]. This includes the Niagara Region of Ontario, where the present study was carried out. Figure 3 shows the impact of latitude on the level of UVB radiation available for cutaneous production of vitamin D, with equatorial regions experiencing year-round vitamin D production, and regions at approximately 40°N latitude and above experiencing some months with no UVB exposure and therefore no vitamin D production [48].

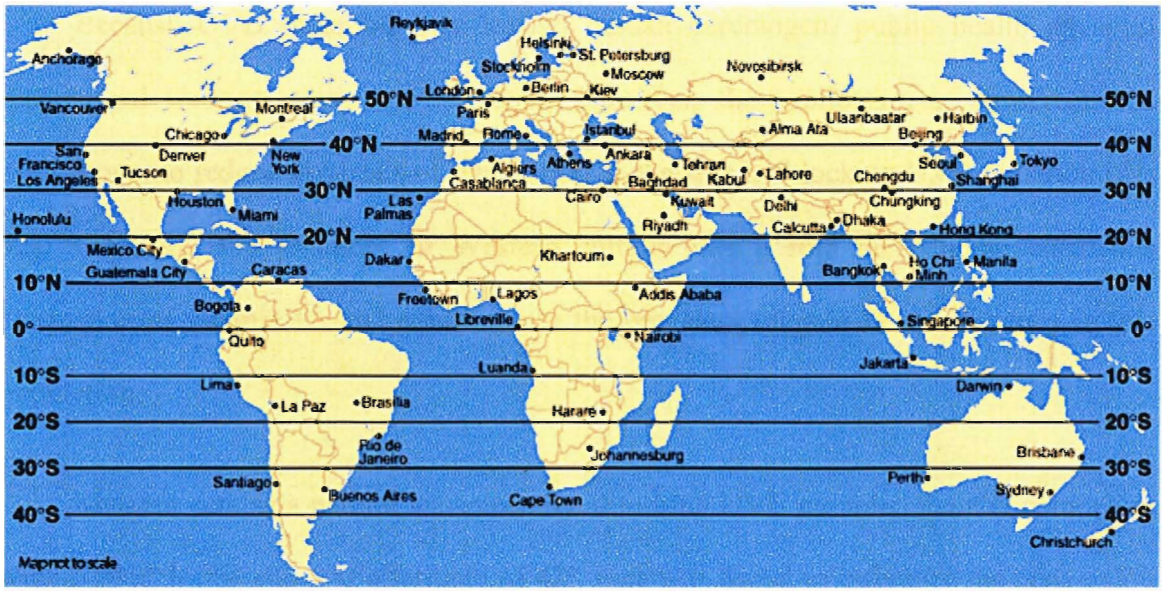


Figure 2: Map of latitude lines and major cities around the world (Taken from: Sky and Telescope, 2008 [50])

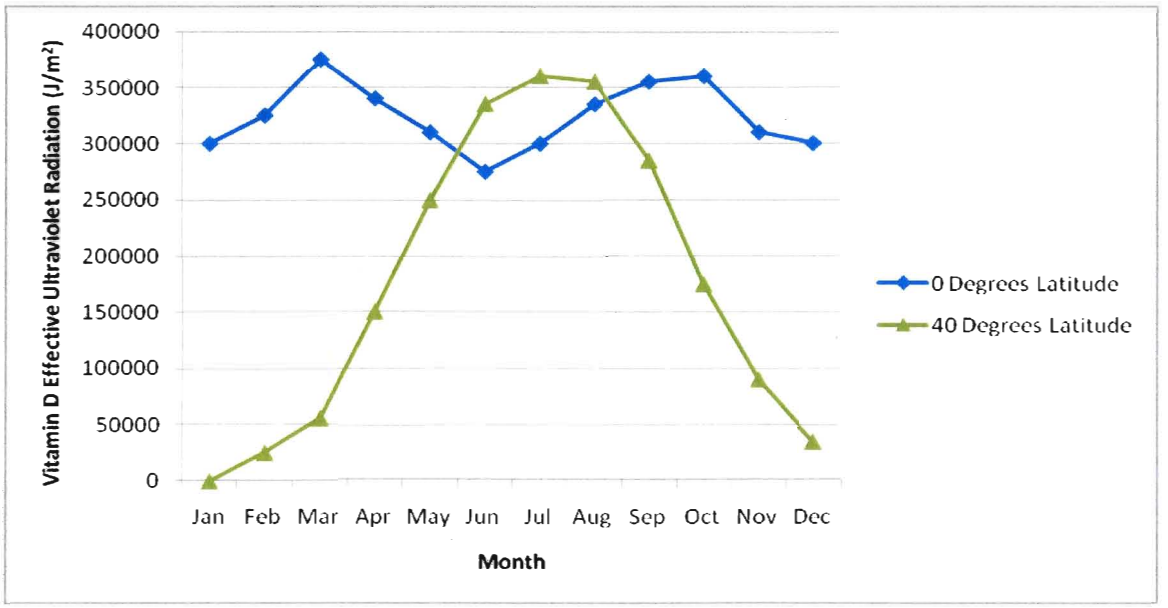


Figure 3: Impact of latitude on the level of UVB radiation available for cutaneous production of vitamin D by month (Adapted from: Kimlin, 2008 [48])

Because UVB radiation is a known human carcinogen, public health agencies recommend wearing sunscreen protection. However, the application of sunscreen has been shown to reduce synthesis of vitamin D_3 in the skin by blocking UVB radiation [51, 52]. Therefore, the practices of Western culture play a role in reducing cutaneous production of vitamin D_3 and contribute to the prevalent vitamin D insufficiency in this population.

Melanin acts as a natural sunscreen by absorbing UVB photons, thereby competing with 7-DHC in the skin. At a latitude of 42°, race was found to influence the conversion of 7-DHC to pre-vitamin D_3, as Whites experienced 5-10 fold greater conversion than Blacks [43]. As well, an *in vitro* study by Liu and colleagues [14] showed that the serum 25(OH)D concentration of Blacks (approximately 23 nmol/L) was much lower than that of Whites (approximately 78 nmol/L). Therefore, individuals with darker skin must spend longer periods of time in the sun in order to endogenously produce the same amount of vitamin D_3 as lighter skinned individuals [49]. Migration of dark-skinned individuals to countries at higher latitudes leads to a reduced cutaneous production of vitamin D_3, an effect that is exacerbated during the winter months [29]. In order to produce sufficient vitamin D_3, Blacks may require 5-50 fold more time exposed to UVB radiation than Whites [23].

In addition to sun exposure, endogenous vitamin D_3 production also depends on the concentration of 7-DHC in the skin. The concentration of 7-DHC has an inverse relationship with age [23, 49], which account for the finding that the continuous vitamin D production of a 70 years old is approximately four fold less than that of a 20 year old

[44]. Similarly, MacLaughlin and Holick [53] found a 50% reduction in 7-DHC in individuals 80 years of age when compared to 20 year olds. This results in circulating concentrations of 25(OH)D among elderly individuals that are 30% of those of young adults [49]. This is the reason Health Canada has recommended that the daily vitamin D intake of adults older than 50 years be twice that of younger adults, although this may not be adequate [54]. A recent report released by the Public Health Agency of Canada (PHAC) [55] on the impacts of falls among the elderly has stated that adults 65 years and up should actually be consuming at least 800 IU vitamin D per day.

2.3 Normal Levels of Circulating Vitamin D

Measures of serum 25(OH)D concentrations are used most often to determine the vitamin D status of individuals. It is very difficult to determine the circulating 25(OH)D concentration cut-points that classify "normal" or "sufficient" vitamin D status, and therefore those concentrations that would indicate a deficient or insufficient status.

Researchers have asserted that determining sufficient circulating concentrations of 25(OH)D should acknowledge that humans have not evolved in an environment of sun avoidance [56]. An optimal range should therefore be based on the concentration of individuals who are continuously exposed to high UVB or living close to the equator, without being overly clothed and without sunblock protection. For that reason, the 25(OH)D concentrations of fieldworkers, construction workers and sunbathers have been recommended for establishing an optimal circulating 25(OH)D range for humans [47, 57]. Individuals who spend their days unprotected from UVB radiation have been reported as

having circulating 25(OH)D concentrations ranging from approximately 100-200 nmol/L [56].

A consensus as to what the maximum serum concentration of 25(OH)D should be has not been reached. At a recent round table discussion in 2005 [58], leading experts recommended that optimal concentrations of circulating 25(OH)D should be maintained at a minimum of approximately 75-80 nmol/L. Cut-points for establishing deficient, insufficient and sufficient concentrations of serum 25(OH)D have been determined by evaluating biomarkers of the biological function of vitamin D and its homeostasis with calcium, such as calcium absorption, parathyroid hormone (PTH) production, BMD and bone fractures. Table 3 provides an outline of how these factors have been used to establish "sufficient" and "deficient" cut-points for vitamin D status.

2.3.1 The Role of Parathyroid Hormone in Establishing Cut-Points

When evaluating 25(OH)D cut points, an important factor to take into consideration is that increasing age has a significant effect on the body's vitamin D requirement. Among the elderly, secondary hyperparathyroidism is an indicator of poor vitamin D nutritional status, as the inverse relationship between PTH and serum 25(OH)D concentrations becomes more pronounced with age [47]. This inverse relationship ensures that as serum 25(OH)D concentrations begin to fall, PTH begins to be synthesized and secreted. Interestingly, this effect begins to subside as serum 25(OH)D concentrations approach 75-80 nmol/L, and PTH levels reach their optimal physiological concentrations and plateau [47, 56, 59, 60].

2.3.2 The Role of Calcium Absorption and BMD in Establishing Cut-Points

Calcium absorption may also act as a functional end point when determining optimal concentrations of circulating 25(OH)D. At a 25(OH)D concentration of 80 nmol/L, calcium absorption reaches a threshold, but begins to decrease as 25(OH)D concentrations drop below 80 nmol/L [47, 56]. Calcium absorption may be an important factor in determining vitamin D deficiency cut points. The presence of bone diseases, such as rickets among the young and osteomalacia among adults, are significantly increased when 25(OH)D concentrations are below 12.5 nmol/L, although levels maintained at less than 25 nmol/L over long time periods may also increase disease development [23, 57]. Many clinicians, assay manufacturers and clinical chemical laboratories, recognize the cut-point of 25(OH)D deficiency as being approximately 25 nmol/L [47]. Table 3 provides a summary of cut-points, as determined by the analysis of PTH concentrations, calcium absorption and bone fractures.

Table 3: Cut-points of circulating 25(OH)D concentrations established by various researchers, and the cut point justifications*

Cut Point level of 25(OH)D**	Explanation	Reference
≥80 nmol/L (sufficient) <22.5 nmol/L (deficient)	• 96% of women with hip fractures have circulating 25(OH)D <80 nmol/L • One year after fracture event, those with levels <22.5 nmol/L have higher fall rates and poorer tests for the performance level of lower extremities	LeBoff et al, 2008 [61]
~100 nmol/L (sufficient)	• Prevention of hip and all non-vertebral fractures with 25(OH)D levels of 100 nmol/L	Bischoff-Ferrari et al, 2005 [62]
90-100 nmol/L (sufficient)	• BMD continued to increase among young White and Mexican American individuals with circulating 25(OH)D levels >100 nmol/L	Bischoff-Ferrari et al, 2004 [63]
37.5 nmol/L (deficient) <12.5 nmol/L (severely deficient)	• Serum PTH levels start increasing if circulating 25(OH)D levels decrease below 37.5 nmol/L	Arya et al, 2004 [64]
~80 nmol/L (sufficient) ~50 nmol/L (deficient)	• Intestinal calcium absorption significantly increased by ~65% with an increase of 25(OH)D levels from 50 nmol/L to 80 nmol/L	Heaney et al, 2003 [65]
75-80 nmol/L (sufficient)	• Optimal calcium absorption • PTH levels elevated in only 10% of the participants	Holick et al, 2005 [66]
<20 nmol/L (deficient)	• Obvious signs of deficiency occur, such as bone pain and reduced BMD	Moore et al, 2005 [67]
~80 nmol/L (sufficient)	• Risk of all cancers was reduced 35% among healthy, postmenopausal women	Lappe et al, 2007 [38]
80-90 nmol/L (sufficient) 37.5 nmol/L (deficient)	• Risk of colorectal cancer was 46% lower among women with 88 nmol/L versus 37.5 nmol/L [25(OH)D]	Feskanich et al, 2004 [68]
100 nmol/L	• Risk of multiple sclerosis was 62% lower, particularly when measured in young women <20 years	Munger et al, 2006 [69]

* Serum 25(OH)D concentration cut points classify vitamin D status as sufficient, insufficient or deficient
** Serum 25(OH)D concentration is converted from nmol/L to ng/mL by dividing by 2.496

2.4 Detection of Vitamin D Metabolite Concentrations

Assuring adequate levels of vitamin D is essential for overall health. This requires the accurate measurement of vitamin D metabolite concentrations. However, vitamin D determination is hindered by various challenges. As previously stated, vitamin D status is most often established by measuring circulating 25(OH)D concentrations. Although $1,25(OH)_2D$ concentrations may also be measured to analyze vitamin D status, assays quantifying the hormonally active metabolite have not been as widely evaluated [70] and serum concentrations of $1,25(OH)_2D$ may not be a reliable measure of vitamin D status. This is because it is less abundant in circulation and has a shorter half-life than its precursor 25(OH)D, which makes up approximately 94% of circulating vitamin D metabolites and has a half-life of about three weeks [49, 71]. Conversely, the half-life of $1,25(OH)_2D$ is about 4-6 hours [71], and it circulates at concentrations about 1000 times lower than 25(OH)D [59].

Hepatic production of 25(OH)D is primarily dependent on the availability of vitamin D, and is not strongly regulated by other factors [72]. In contrast, $1,25(OH)_2D$ concentrations may fluctuate according to parathyroid hormone (PTH) concentrations. During vitamin D deficiency, intestinal calcium absorption is reduced, which triggers the production and secretion of PTH from the parathyroid glands. In turn, PTH increases calcium resorption and renal $1,25(OH)_2D$ production. Therefore, vitamin D deficient individuals with normal renal function may show either normal or elevated levels of $1,25(OH)_2D$ [45]. 25(OH)D is the vitamin D metabolite that was measured and analyzed in the present study, and is a measure of the exposure and supply of the nutrient to target

tissues. The following describes the gold standard test for measuring circulating 25(OH)D concentrations, as well as the assay utilized in the present study and the rationale for its use.

2.4.1 The Gold Standard Test

In 1977, the high performance liquid chromatography (HPLC) method of directly detecting circulating 25(OH)D was introduced. HPLC allows for large amounts of crude serum to be analyzed in a short amount of time with high specificity and sensitivity, and is considered to be the gold standard test [73]. HPLC is used to separate the components of a mixture, in this case 25(OH)D_2 and 25(OH)D_3, for identification and quantification by UV detection methods. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is employed as well, which utilizes mass spectrometry to quantify vitamin D levels [74]. It is also able to separate and accurately quantify 25(OH)D_2 and 25(OH)D_3 [75]. These methods are not always available to clinical laboratories, due to the high financial costs involved, and need for highly trained and experienced personnel to operate the instruments [76]. This is why simpler, more cost-effective methods are required.

2.4.2 Radioimmunoassay

The principle behind radioimmunoassays (RIA) involves the ability of high-affinity anti-antigen antibodies to competitively bind with a known amount of radioactively-labelled antigen and unlabelled antigen [77]. The radioisotope ^{125}I is the most commonly used when performing a RIA, because iodination of proteins occurs without the disruption of their immune specificity [74]. Separation of the 25(OH)D-antibody complex allows for

the amount of labelled 25(OH)D-antibody to be measured. A large amount of labelled 25(OH)D-antibody indicates that only a small amount of 25(OH)D is available in the serum sample to compete, and therefore the serum 25(OH)D concentration in the sample is small. Conversely, a small amount indicates that a large concentration of 25(OH)D is present in the serum sample. Gamma radiation of ^{125}I-labelled 25(OH)D-antibody is measured and quantification of the 25(OH)D concentration is determined by a standard curve [77].

2.4.3 Choosing a Method to Measure Serum 25(OH)D Concentration

Since 1989, all methods of determining vitamin D status have been regularly analyzed by the Vitamin D External Quality Assessment Scheme (DEQAS), in order to improve the precision and accuracy of available assays [78]. Two of the main 25(OH)D assays being tested and utilized within the research community are the chemiluminescent competitive protein-binding Nichols Advantage® 25-OH Vitamin D (developed by Nichols Institute Diagnostics) and the DiaSorin 25-OH Vitamin D Radioimmunoassay® (developed by DiaSorin). The main difference between the two assays is that DiaSorin RIA® uses an antibody to 25(OH)D, whereas Nichols Advantage® uses a DBP [79].

A study by Binkley and colleagues [80] looked at the variation between assays and the potential to confound diagnosis in a clinical setting. The study assigned serum samples to eight different labs, each having to establish 25(OH)D concentrations by a specific method. Three labs used the Nichols Advantage® assay, two implemented the DiaSorin RIA®, and one used the gold standard HPLC (two labs used other methods). The study

showed substantial variation in the serum 25(OH)D concentrations determined by different labs using the same and different methods. The proportion of individuals falling below the pre-determined sufficiency cut-point of 80 nmol/L varied by 17-90%. The Nichols Advantage® assays all produced overestimated values of the basal 25(OH)D concentrations and underestimations of exogenously added $25(OH)D_2$. The Nichols Advantage® assay yielded a poor comparison to the HPLC method, while DiaSorin RIA® showed a good comparison with the gold standard HPLC in the study. Similar results were obtained by Terry and colleagues [79], who found that the Nichols Advantage® yielded values less than 40% the values of the gold standard.

DEQAS has repeatedly reported that $25(OH)D_2$ and $25(OH)D_3$ are detected accurately with the DiaSorin RIA® assay. Conversely, DEQAS suggests that Nichols Advantage® competitive protein-binding assay is not capable of properly detecting $25(OH)D_2$, and frequently leads to underestimations of total 25(OH)D concentrations, as both metabolites are found in serum [70, 73, 81]. This is in contrast to the manufacturer's labelling, which claims that Nichols Advantage® will have 100% cross-reactivity between $25(OH)D_2$ and $25(OH)D_3$. As a result, many studies report that the Nichols Advantage® assay shows a significant discrepancy with the gold standard HPLC [70, 73, 76], and is the reason it has not been implemented in the present study.

Discrepancies between DiaSorin RIA® testing laboratories have highlighted the fact that a lack of experience with the assay, and a lack of validation within the lab can likely lead to variable and unreliable results [73]. In the present study, maintaining reliable results with the DiaSorin RIA® was ensured by the use of quality control measures. For

example, assay performance was monitored using two controls, at a low-normal concentration and at a high-normal concentration, which were provided by the assay kit manufacturers. As well, the controls, along with the unknown samples and standards were assayed in duplicate. Intra-assay coefficient of variation (CV) (calculated by dividing the mean 25(OH)D concentration by the standard deviation) greater than 10% required re-assaying. Similarly, sample 25(OH)D concentrations above 70 ng/mL (175 nmol/L) or the lowest standard 5.0 ng/mL (12.5 nmol/L) were verified by re-extraction and re-assay (samples with concentrations above 100 ng/mL (250 nmol/L) were diluted before re-extraction).

2.5 Vitamin D Metabolism

As previously discussed, vitamin D can be taken in by diet or exposure to UVB radiation. Once it is ingested, dietary vitamin D is absorbed in the intestines. Since vitamin D is a fat-soluble molecule, it must be consumed with lipids for proper absorption. Dietary vitamin D is absorbed from a micelle with fat, and broken down with bile salts. It is then able to passively diffuse into the cells of the small intestines, where most of the vitamin D consumed in the diet is absorbed. Intestinal cells contain chylomicrons, lipoproteins that transport about 40% of the dietary vitamin D to the lymphatic system and blood stream [30].

Once vitamin D has been ingested and absorbed, or cutaneously produced and diffused through the skin, it enters circulation bound to DBP [30, 49]. Approximately 60% of vitamin D travels in serum bound to DBP [30]. Albumin and lipoproteins also

transport vitamin D in the blood [82]. Vitamin D is primarily carried to the liver, although some is taken to extrahepatic tissues by DBP [30].

As depicted in Figure 4, vitamin D undergoes two important hydroxylation reactions in order to become the biologically active hormonal form. The first of these reactions takes place in the mitochondria of liver cells. Mitochondrial cytochrome P450 enzyme 25-hydroxylase (also called P450C25 or CYP27A1) hydroxylates the vitamin D molecule at carbon 25, producing 25-hydroxyvitamin D [25(OH)D]. This molecule is biologically inert; however it is the major form in which vitamin D circulates, and its serum concentrations peak approximately three hours following UVB exposure [49, 83, 84]. Although 25-hydroxylase is most abundant in the liver, it is also found in extrahepatic tissues such as the lungs, intestines, kidneys and skin, suggesting that 25(OH)D may also be produced outside of the liver [30, 49].

The production of 25(OH)D is regulated, though poorly, by a negative feedback mechanism controlled by circulating concentrations of vitamin D, 25(OH)D, and $1,25(OH)_2D$. For example, high concentrations of 25(OH)D may inhibit the production of 25-hydroxylase, thereby preventing further production of 25(OH)D. Also, high concentrations of $1,25(OH)_2D$ can inhibit production of 1α-hydroxylase [responsible for the hydroxylation of 25(OH)D to $1,25(OH)_2D$], which then increases 25(OH)D levels in the blood (Figure 4) [49, 59]. 25(OH)D is not stored in the liver, and is quickly released into circulation, where it has a half-life of approximately three weeks, and travels to the kidneys bound to DBP [29, 57].

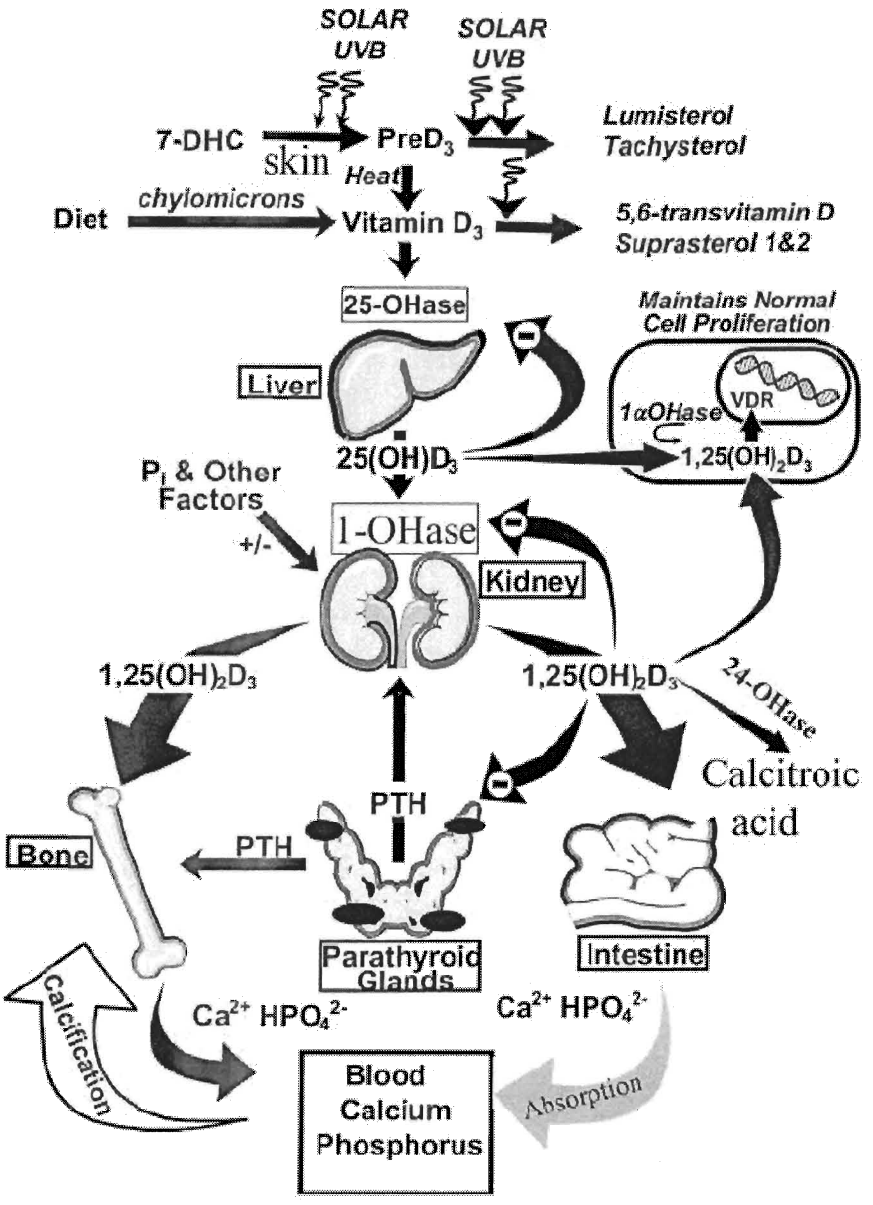


Figure 4: The cutaneous production and dietary absorption of vitamin D, and its subsequent metabolism and regulation (25-OHase, 25-hydroxylase; 1-OHase, 1α-hydroxylase) [59]

Inside the kidneys, 25(OH)D undergoes hydroxylation into the biologically active metabolite. Once the DBP-25(OH)D complex reaches the kidneys, it is translocated by

megalin to the proximal and distal renal tubules [29, 84]. Megalin is a low-density lipoprotein receptor on the proximal tubular epithelium of the kidneys, and mediates the uptake of DBP-25(OH)D complexes into the tubules [85]. Figure 5 shows that the DBP-25(OH)D complex binds to the megalin receptors, which mediate entrance into the proximal tubule cells. The DBP protein is then degraded in the lysosome, and 25(OH)D is carried by intracellular vitamin D binding protein 3 (IDBP-3) to the mitochondria, where it undergoes hydroxylation by the cytochrome P450 enzyme 1α-hydroxylase (also called P450C1 or CYP27B1) to form $1,25(OH)_2D$. Alternatively, some 25(OH)D molecules will re-enter circulation and bind to DBP [49, 86, 87]. Although the kidneys are the primary site of $1,25(OH)_2D$ production, extra-renal production takes place in more than thirty tissues, such as placental cells, bone cells, keratinocytes, macrophages, and intestinal cells [29, 30, 49, 57].

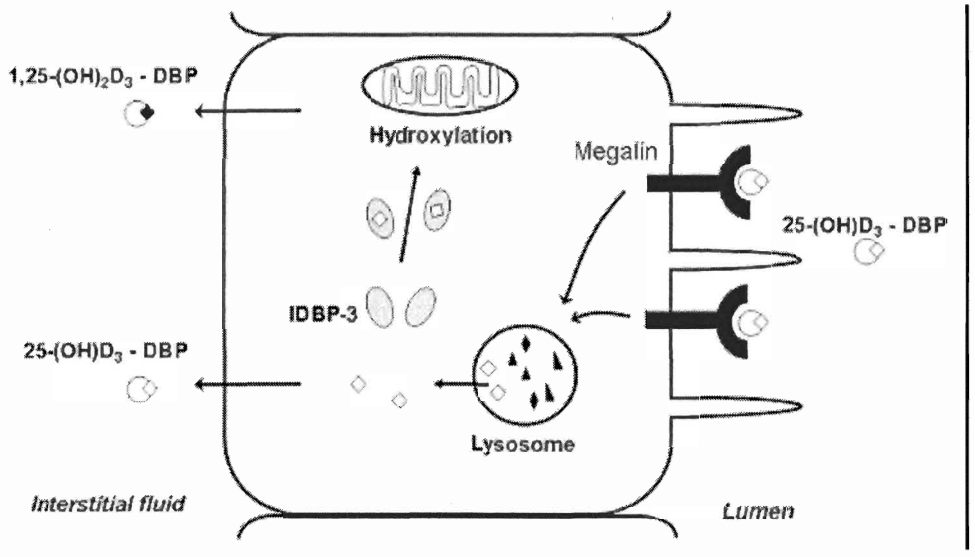


Figure 5: The role of megalin and IDBP-3 in the hydroxylation of 25(OH)D in the kidneys [87].

The regulation of the active vitamin D metabolite, $1,25(OH)_2D$, is primarily controlled by the expression of the 1α-hydroxylase enzyme, which is stimulated by PTH, low plasma calcium concentrations and calcitonin, and inhibited by dietary phosphorus and high $1,25(OH)_2D$ concentration [30, 84]. This may be partially explained by the ability of PTH to increase the expression of vitamin D receptor (VDR) [88] and calcium-sensing receptor proteins in the kidneys (responsible for monitoring changes in calcium concentrations) [84]. Additionally, high concentrations of $1,25(OH)_2D$ down-regulate 1α-hydroxylase expression in the kidneys, and increases tubular and intestinal calcium absorption [57].

Also aiding in the regulation of $1,25(OH)_2D$ is the presence of other vitamin D metabolites. More than forty other metabolites may be formed when 25(OH)D is hydroxylated (by various hydroxylases), all of which are less biologically active than $1,25(OH)_2D$. For example, 24-hydroxylase can convert 25(OH)D into $24,25\text{-}(OH)_2D$, and $1,25(OH)_2D$ into $1,24,25\text{-}(OH)_3D$ [30, 57]. This enzyme aids in the regulation of $1,25(OH)_2D$ by converting it to the less active $1,24,25(OH)_3D$ form [89], and is expressed in most cells, though primarily in the proximal tubule of the kidneys [84].

$1,25(OH)_2D$ carries out its hormonal functions on target cells by interacting with VDR. From the kidneys, $1,25(OH)_2D$ is released into circulation loosely bound to DBP, so it may be easily released to the VDR at target tissues. The half-life of $1,25(OH)_2D$ is approximately 4-6 hours [30], and the half-life of DBP is about 2.5 days [83]. Interestingly, although DBP binds 25(OH)D 668 times more strongly than $1,25(OH)_2D$, $1,25(OH)_2D$ binds to VDR 668 times more strongly than 25(OH)D [86]. This ensures the

25(OH)D is the primary metabolite in circulation, while $1,25(OH)_2D$, the biologically active form, is able to carry out its functions within target cells.

Circulating 25(OH)D is the form in which vitamin D is primarily stored. Vitamin D can also be stored in adipose tissue, where it has a half-life of approximately eighty days, and therefore may be used during winter months or times of low vitamin D production or consumption [83]. However, studies have found that obese individuals (body mass index (BMI) $\geq$30 kg/m^2) are more likely to have lower serum 25(OH)D concentrations than people of normal weight, possibly because vitamin D is sequestered deeper in fat stores, making it less available for hydroxylation into 25(OH)D [29, 90-92]. Wortsman and colleagues [93] reported more than 50% reduction in bioavailability of cutaneously produced vitamin D in obese individuals, but no significant difference in the peak circulating vitamin D following supplementation, indicating that the inverse relationship is likely due to an inability to maximally absorb cutaneously produced vitamin D.

2.6 Mechanisms of Action

$1,25(OH)_2D$ may act through both genomic and non-genomic pathways when carrying out its functions. The $1,25(OH)_2D$-DBP complex is taken up by the cell from circulation through diffusion or receptor-mediated endocytosis. Once inside, $1,25(OH)_2D$ is released from DBP and forms a complex with the vitamin D receptor (VDR). The VDR is the $1,25(OH)_2D$ receptor, mediating its effect on the target cell [84]. It has been hypothesized that a non-genomic $1,25(OH)_2D$-mediated mechanism of action can induce a more rapid response from the cell. These mechanisms involve the association of

$1,25(OH)_2D$ with a membrane VDR that activates signal transductions such as calcium transport channels and mitogen-activated protein kinase (MAPK) activation [86, 94]. Association with cytoplasmic plasma membrane proteins such as tyrosine kinase receptor or G protein coupled receptor [84, 94] may lead to phosphorylations and involvement of secondary messengers that carry out rapid responses in the liver, intestines, bone, parathyroid and pancreatic β-cells [30].

Genomic pathways take place as the $1,25(OH)_2D$-VDR complex moves from the cytosol into the nucleus [82]. By binding to VDR, $1,25(OH)_2D$ is able to regulate the transcription rates of more than sixty genes involved in mechanisms such as calcium balance, immune regulation and cell differentiation [83]. The binding of $1,25(OH)_2D$ to VDR leads to conformational changes and phosphorylations that cause the $1,25(OH)_2D$-VDR complex to heterodimerize with retinoid X receptor (RXR), a nuclear hormone receptor [95].

VDR is a member of the nuclear hormone receptor superfamily, and has two zinc fingers that allow for interaction with specific vitamin D response elements (VDRE) in the promoter regions of various genes [84, 94]. VDREs mediate $1,25(OH)_2D$ -dependent gene expression (Wang 2004). Co-modulatory proteins (co-activators or co-repressors) also interact with the VDR-RXR complex to enhance or repress gene transcription [30]. An example of a co-activator is VDR-interacting protein (DRIP), which recruits RNA polymerase II holoenzyme [88], and may also be responsible for unfolding and exposing DNA for transcription [82]. These types of mechanisms may potentially take place in any of the more than 30 tissues in which nuclear VDRs have been isolated, including bones,

intestines, kidneys, lungs, muscle and skin [30]. Biological actions resulting from these reactions include calcium homeostasis, mediation of $1,25(OH)_2D$ synthesis and breakdown rates, reduction in PTH production and regulation of immune responses [82].

2.7 Biological Functions

There is a large body of evidence for the role for vitamin D in calcium metabolism and PTH balance. The association between vitamin D and bone metabolism has also been observed in studies of VDR gene polymorphisms and the development of osteoporosis [96], rickets [16] and occurrence of hip fractures [61]. However, the involvement of vitamin D in the immune processes is less well understood. This section will provide a brief review of the relationship between vitamin D and immune reactions, and then discuss specific diseases associated with vitamin D deficiency.

2.7.1 Involvement of Vitamin D in Immune Processes

The immunomodulatory role of $1,25(OH)_2D$ is primarily due to the local production of the hormone by immune cells such as macrophages, dendritic cells (DCs) and activated lymphocytes, attenuating adaptive and enhancing innate immune mechanisms [97]. The production of VDR in these cells allows $1,25(OH)_2D$ to regulate expression of cytokines and cell receptors [1].

Vitamin D metabolites primarily regulate innate immunity by interacting with macrophages, which express 1-hydroxylase, the enzyme responsible for converting 25(OH)D into the biologically active $1,25(OH)_2D$ [97]. Macrophages are cells that are

commonly exposed to pathogens, recognizing and responding to pathogen-associated molecular patterns (PAMPs) on the cell surface of microbes [98]. The PAMPs are recognized by pattern recognition receptors (PRRs) on the cell surface of macrophages [99]. An important group of PRRs are Toll-like receptors (TLRs), differing in their abilities to recognize certain groups of pathogens. The most important vitamin D-associated innate immune response to infection is the release of the antimicrobial protein cathelicidin [100, 101]. In response to pathogenic antigens, macrophages produce the biologically active $1,25(OH)_2D$ from circulating 25(OH)D and up-regulate expression of cathelicidin, thereby increasing pathogen killing [14].

As well as its role in increasing the innate immune reactions, $1,25(OH)_2D$ plays a significant role in adaptive immunity. Macrophages and dendritic cells (DC) act as antigen presenting cells (APC), presenting antigens to resting T or B lymphocytes and activating them [97] (Figure 6). Activated T and B lymphocytes both express VDR, and therefore allow for the effects of $1,25(OH)_2D$, such as suppressing the proliferation of B and T lymphocytes and reducing immunoglobulin production. The primary target is the T helper (Th) cell, which modulates cytokine production [97]. T helper lymphocytes differentiate into Th1 and Th2 lymphocytes. This polarization of the adaptive immune system influences production of various cytokines. Interleukin (IL)-12, produced by macrophages and DCs, differentiates naive Th lymphocytes into Th1 lymphocytes [98]. $1,25(OH)_2D$ inhibits IL-12 expression by macrophages and DCs [102]. This is consistent with the commonly acknowledged role of $1,25(OH)_2D$ in immunosuppression, as the hormone has been shown to suppress the Th1 pathway and induce the Th2 pathway,

inhibiting inflammatory cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-2, and increasing development of Th2 cells and corresponding cytokines (IL-4, IL-5, IL-10 and IL-13) [1, 103]. This type of reaction is beneficial in combating the symptoms of some chronic conditions, such as autoimmune or inflammatory diseases [104]. However, the mechanism by which it aids in fighting some intracellular pathogens has not been as well described.

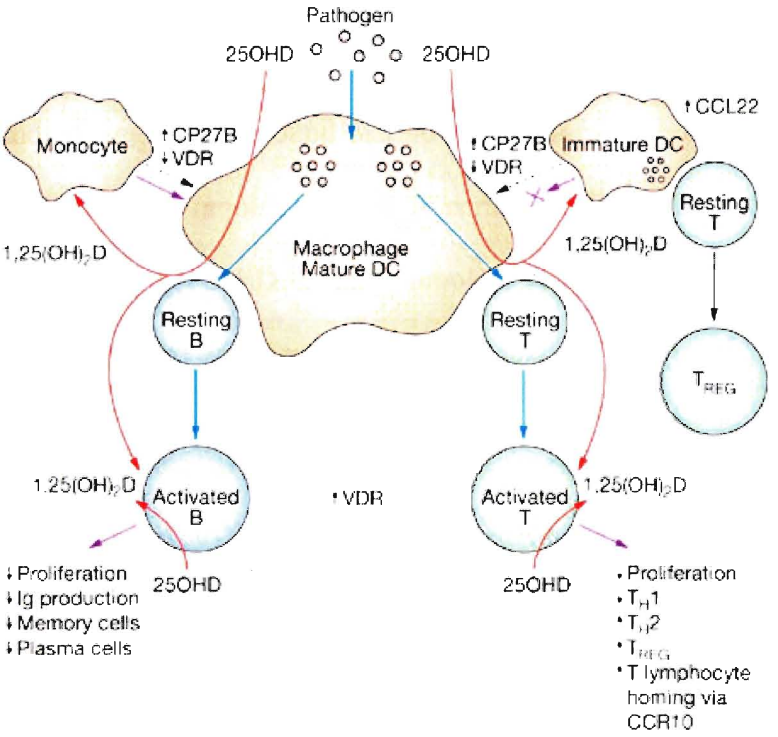


Figure 6: Interactions between vitamin D metabolites and the immune system [97]

2.7.2 Vitamin D Deficiency and Disease Processes

As emphasized thus far, vitamin D plays an important role in maintaining several biological functions. In absence of sufficient circulating 25(OH)D concentrations, there is potential for disease to develop. The following is a brief description of some chronic and infectious disease-related implications of vitamin D deficiency.

2.7.2.1 Chronic Conditions

One of the primary negative effects of vitamin D deficiency is the decrease in BMD. Studies of BMD are commonly carried out among post-menopausal women [105]. For example, LeBoff and colleagues [61] found that 96% of women with hip fractures had a vitamin D insufficiency (<32 nmol/L 25(OH)D concentration), 38% of whom had concentrations of circulating 25(OH)D below 9 nmol/L. As well, VDR polymorphisms have been found to be associated with vertebral fractures [96].

Vitamin D deficiency has also been shown to have an association with various types of cancer. For example, certain VDR gene polymorphisms have been associated with an increased risk of colon cancer among white individuals [106]. Animal studies have suggested that an increase in either exogenous or endogenously-produced vitamin D intake may be preventative against colon cancer. This may be attributed to the effect of $1,25(OH)_2D$ in reducing the inflammatory processes associated with conditions such as inflammatory bowel disease and Crohn's disease, which have been shown to be predisposing factors for colon cancer [107, 108]. Additionally, a recent meta-analysis found that women consuming less than 400 IU/day were more likely to develop breast

cancer [109]. Vitamin D deficiency has also been linked to mortality. For example, improved vitamin D and calcium nutrition was associated with a reduced rate of all-cancer mortality [38]. As well, Melamed and colleagues [110] found that vitamin D deficiency (<45 nmol/L 25(OH)D concentrations) may be associated with a 26% increase in the risk of all-cause mortality.

2.7.2.2 Infectious Diseases

In contrast with chronic health conditions, the role of vitamin D in infectious diseases has not been as widely established, although recent studies have shown significant associations between vitamin D and some infectious diseases. For example, reduced levels of 25(OH)D and $1,25(OH)_2D$ have been found among groups of HIV-positive individuals, compared with HIV-negative individuals [111]. In addition, HIV-positive patients are more likely to have osteoporosis and osteopenia, conditions commonly associated with vitamin D deficiency and successfully treated with vitamin D supplements [112]. Contrary to the previous section discussing the immunosuppressive nature of $1,25(OH)_2D$, a small cohort of HIV-infected individuals suggested a relationship between high concentrations of $1,25(OH)_2D$ and reduced mortality, due to a positive association between $1,25(OH)_2D$ concentrations and CD4+ T cells counts [111]. This relationship is not well described, and remains to be further studied.

Studies in India have found an association between respiratory tract infections among children and insufficient concentrations of circulating 25(OH)D. For example, one hospital-based case-control study reported that severe acute lower respiratory infections,

primarily pneumonia, occurred more frequently among vitamin D deficient children under the age of five years [113]. Also infecting the respiratory tract, mycobacteria are intracellular organisms commonly found to be affected by vitamin D deficiency. $1,25(OH)_2D$ has been shown to enhance the intracellular killing of *Mycobacteria bovis* by increasing the production of antimicrobial peptides in macrophages, such as cathelicidin [101, 114]. The infectious agent most commonly associated with vitamin D deficiency is *M. tuberculosis*. The association between the hormone and bacterium will be the focus of the present study, and the following section will provide an introduction to the characteristics of the pathogen and its disease process.

2.8 Tuberculosis

Before going into detail about the association between vitamin D and tuberculosis, the global epidemiology and characteristics of TB will be briefly discussed.

2.8.1 Global and Canadian Epidemiology of TB

Reported by the WHO to infect approximately one third of the global population [4], *M. tuberculosis* continues to pose a major threat to a large proportion of the world, as about nine million new cases of the disease are reported each year (90% of which occur in developing countries) [115], and about two million individuals die of the disease [116]. Incidence rates of more than 300 new cases per 100,000 population continue to be the highest in sub-Saharan African countries, and the lowest incidence rates (0-24/100,000) in North America, Western Europe, and Australia [116]. The WHO's Millennium

Development Goal 6, to halve the 1990 prevalence and death rates of TB by 2015, may be achievable, as most of the world has reported decreasing incidence rates [116].

In 2005, 1616 cases (5/100,000) of new active and relapsed TB were reported in Canada, ranging from an incidence rate of 0.6/100,000 in Nova Scotia to 150/100,000 in Nunavut. Cumulatively, British Columbia, Ontario and Quebec make up approximately 75% of the Canadian population, and report 71% of the total number of reported TB cases. The largest proportion of reported cases occurs among individuals aged 25-34 years (17% reported cases) and those who are foreign-born (63% reported cases). Canadian-born Aboriginals make up 19% of total cases reported and Canadian-born non-Aboriginals 13%. Pulmonary TB is the most common site of diagnosis (57% cases) [117].

2.8.2 Characteristics of Mycobacterium tuberculosis

Mycobacterium tuberculosis was described in 1882 by Robert Koch as the causative agent of TB [118]. The *Mycobacterium* genus belongs to Group II of the branching, gram-positive bacterial family Mycobacteriaceae, and consists of more than 85 species [118, 119]. The three species that most commonly cause human disease are *M. tuberculosis*, *M. leprae* and *M. ulcerans* [120].

M. tuberculosis are facultative intracellular bacilli with hydrophobic cell walls rich in mycolic acids [98]. These complex, branched-chain hydroxyl lipids are characteristic of the *Mycobacterium* genus, and allow for the distinctive staining property of acid-fastness, as mycolic acids react with the red carbol fuchsin dye [118]. Sixty percent of the cell wall weight is made up of lipids. The resulting low permeability of the cell wall contributes to

the natural resistance of the bacilli to regular antibiotics [120]. Individual *M. tuberculosis* cells are approximately 0.5 μm in diameter and have high-GC genomes. *M. tuberculosis* is an extremely slow-growing organism with a replication time of approximately 30 hours, taking from days to weeks to produce colonies in artificial cultures [118].

2.8.3 Outcomes of M. tuberculosis Infections: Disease, Latency and Reactivation

Primary exposure to *M. tuberculosis* occurs by the inhalation of droplet nuclei containing the bacteria. However, a complex network of predisposing environmental and genetic factors determine whether the infection will establish and progress to active TB disease (10% of known infections), lay in a dormant state as LTBI (90% of known infections), or clear from the body without establishing an infection or leaving any detectable immune memory [15, 121]. When active disease develops, it most often occurs as pulmonary TB, with extrapulmonary disease manifestations occurring in approximately 21% of cases, according to the WHO [122]. If left untreated, those who develop active pulmonary disease may stabilize and become asymptomatic carriers, or develop disseminated infection that could affect tissues such as the meninges, abdomen, bones and joints, and peripheral lymph nodes [123]. Fibrosis and scarring, resulting from caseation, are the major causes of clinical disease in pulmonary TB, as normal tissue function is impaired and leads to pulmonary difficulties [15, 98, 122].

As mentioned above, 90% of infected individuals will develop LTBI, the vast majority of whom will experience continued latency for the rest of their lives. In this state, bacilli can survive for long periods of time without pathological consequences, as long as

immune mechanisms continue to function effectively [98, 124]. Otherwise, improper granuloma maintenance could lead to rupture and spread of the liquefied caseous center, containing tubercle bacilli, to other areas of the lungs, or possible dissemination to other parts of the body [121]. This granuloma-contained reservoir of *M. tuberculosis* infection has the potential to reactivate and lead to active disease [124]. The majority of reactivated cases will present as pulmonary TB with the potential to spread to 10-15 other susceptible people [124, 125]. It follows then, that reactivated cases are the main reservoir for TB transmission. Approximately 5-10% of individuals with LTBI will reactivate, depending on their immunocompetency [122]. Of the approximately nine million cases diagnosed each year, a great proportion is due to reactivation [116].

Aside from biological factors, there are several social circumstances that contribute to the reactivation of LTBI, including a lack of health care, overcrowded living conditions, malnutrition and stress. Each of these has the potential to influence the progression to disease. For example, stress levels are commonly associated with an increased presence of glucocorticoids in the body, which reduce the activation of macrophages, thereby stimulating the reactivation of TB in humans and animals [126, 127].

A meta-analysis [128] of 38 studies from 16 countries, exploring the association between indoor air pollutions and TB, found that tobacco smoke exposure is another factor that is often associated with active TB, the risk of having a positive tuberculin skin test (TST) or death from TB. Smoke likely inhibits the normal functions of alveolar macrophages, as shown by the reduced phagocytic capabilities, levels of pro-

inflammatory cytokines, and levels of TNF-α and NO in smokers, compared to non-smokers. These are all factors that would influence the ability of the immune system to eliminate the bacilli upon infection, as well as inhibit the maintenance of the granuloma [128].

Increased risk of reactivation is also associated with older age. For individuals older than 65 years, the chance of a TB diagnosis increases by a factor of four. However, interpretation of this fact has to be made with caution, because active disease in the elderly can also be caused by increased susceptibility to new infections [129].

It has also been established that an increased risk of LTBI reactivation is commonly associated with immigration, as this population is more often exposed to risk factors such as poverty, need of public health assistance, unemployment and overcrowding [15]. In Canada, more than 60% of the TB cases are diagnosed in foreign-born individuals, and among them, approximately 41% reactivate within the first five years, as shown in Figure 7 [122].

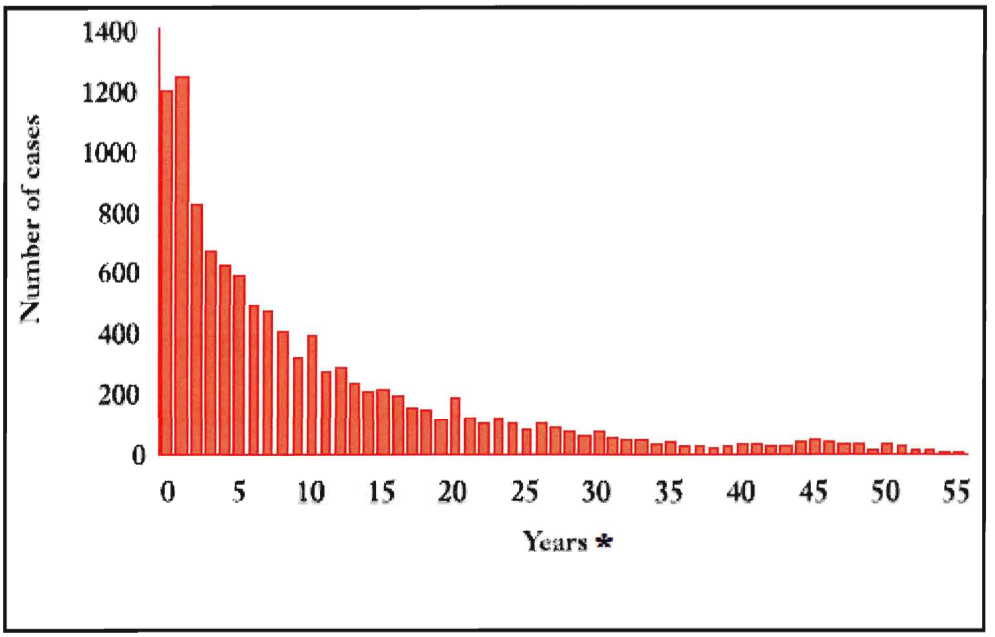


Figure 7: Foreign-born tuberculosis cases in Canada: Time from arrival in Canada to diagnosis in years [122]
*0-55 years post-arrival for 1994-2004 cases

Two studies in Canada used fragment length polymorphism patterns to determine the proportion of current TB cases that resulted from remotely acquired (i.e., reactivation) versus recent infection. One of these studies, by Kulaga and colleagues [130], demonstrated that 82-96% of new active TB cases in Montreal occurred as a result of reactivation. The second study used similar methods to determine that foreign-born individuals in Vancouver, BC, were strongly associated with having LTBI [131].

There are various genetic mutations and polymorphisms that reduce an efficient immune response. More than 100 Mendelian disorders have been identified that increase susceptibility to infection by reducing IL-12 and IFN-γ production [119]. For example, it has been suggested that *VDR* gene polymorphisms contribute to vitamin D deficiency, by reducing effective interactions between $1,25(OH)_2D$ and various immune system

mechanisms. In fact, studies have shown an association between vitamin D deficiency and TB susceptibility [4].

Prevention of LTBI reactivation includes the avoidance or correction of the risk factors mentioned above (excluding the genetic predispositions), as well as the use of preventative therapies, which consist of pharmaceuticals such as isoniazid for 6-9 months or rifampin for four months [132, 133]. However, these drug therapies are avoided unless necessary, due to an increased chance of liver injury [133]. As well, vitamin D deficiency among individuals with TB suggests that vitamin D may be important in preventing reactivation, and may also be a potential form of therapy [11].

2.8.4 Cell-Mediated Immunity Diagnosis of Latent Tuberculosis Infection

Since the focus of the present study is LTBI, providing an in-depth discussion of active TB diagnosis is beyond the scope of this thesis. Briefly, upon clinical suspicion, the diagnosis of active disease involves a combination of clinical history, sputum smear microscopy, bacterial culture and chest radiography [122].

As for LTBI diagnosis, a major problem is the lack of a gold standard, which prevents from establishing the true global prevalence of LTBI [134]. Diagnosis of latent infection is usually determined by the tuberculin skin test (TST), which is based on delayed type hypersensitivity (DTH) reaction, to determine if an individual has previously been exposed to *M. tuberculosis*. TST uses purified protein derivative (PPD), an antigen of *M. tuberculosis*, and its subcutaneous injection evokes a reaction leading to induration within 48-72 hours in those who have been previously exposed [98, 135]. When

interpreting the results of a TST, it is important to consider more than just size of the induration. This is because the technique for measuring the induration in quite variable, and greatly depends on factors such as the experience of the health professional carrying out the test, presence of HIV infection and age [122]. Consequently, there are three dimensions taken into account when evaluating results of a TST. The first is size of the induration, usually considered positive when the induration is $\geq$10 mm. The second dimension is the positive predictive value. TST does not differentiate between LTBI and active disease, and the positive predictive value is reduced by the decreased specificity of the test. For example, positive TST results often occur in people who have been vaccinated by Bacille Calmette-Guérin (BCG) vaccine in the last ten years or have been infected by other environmental mycobacteria. For these reasons, a positive TST may not always be indicative of a current TB infection [122, 135]. Lastly, the third dimension is the risk of developing active TB disease by evaluating risk factors associated with reactivation of LTBI [122]. The specificity and sensitivity of TST are 70-85% and 75-90%, respectively [136].

A newer technology for diagnosing LTBI is the interferon-gamma release assays (IGRAs). IGRAs are cell-mediated immunity diagnostics, measuring the concentration of IFN-γ released in response to TB antigens in whole blood. In Canada there are two FDA-approved commercially available tests based on this principle. They are the T-SPOT.TB® (Oxford, Immunotec) and the QuantiFERON-TB Gold® (Cellestis Limited, Australia). The present study uses the latter method, specifically the QuantiFERON-TB Gold In-Tube®, which uses an enzyme-linked immunosorbent assay (ELISA) to detect the

presence of IFN-γ released by memory T cells upon *in vitro* stimulation with TB-specific antigens, ESAT-6, CFP-10 and TB7.7 [115, 134, 137]. Unlike the TST, IGRA is able to distinguish between LTBI and a BCG-vaccinated individual, as these antigens used in the assay are absent from all BCG strains [134].

In general, IGRAs have shown superior performance to the TST, and are becoming the preferred method to assess LTBI. The specificity and sensitivity of the QuantiFERON-TB Gold In-Tube® method has been reported as 90-100% and 75-95%, respectively [136]. In absence of a gold standard test for LTBI, the IGRA was used to determine LTBI status in the present study.

2.8.5 Immune Response to M. tuberculosis

Following inhalation of the *M. tuberculosis* bacilli, the pathogen is phagocytosed by alveolar macrophages. Various receptors (Figure 8) play a role in the endocytosis of the bacteria. Essential to this are the plasma proteins of the complement system's lectin pathway [98]. Dectin-1, for example, is a C-type lectin that has been shown to cooperate with Toll-like receptor (TLR)-2 in the recognition of *M. tuberculosis* [4]. As well, mannose-binding lectin (MBL) recognizes and opsonises *M. tuberculosis* cell walls at mannose residues or N-acetylglucosamine oligosaccharides of cell wall polysaccharides [4, 121]. Binding these residues triggers the complement system proteins and proteases to initiate phagocytosis through macrophage membrane receptors, such as the mannose receptor (MR) [4, 119], and to act as chemoattractants that stimulate inflammation [98]. Mannose-binding lectin is part of the collectin family of proteins that also includes

surfactant proteins (SP) (also C-type lectins) that maintain alveoli surface tension. Two of these, SP-A and SP-D, play a role in the modulation of macrophage-*M. tuberculosis* interactions, leading to the production of reactive nitrogen intermediates (RNI), lung inflammation and antibacterial actions within the macrophage [4]. CR3 is a complement receptor that plays a large role in phagocytosis, and in its absence, *M. tuberculosis* phagocytosis by macrophages may be reduced by 70-80% [121].

The transmembrane TLR-2 homodimerize or heterodimerize with TLR-6 or TLR-1 (Figure 8), and act as pattern recognition receptors of lipopeptides, mycolylarabinoglycan-peptidoglycan complex (cell wall component), lipids and lipoarabinomannan (LAM) (cell wall component) of *M. tuberculosis*. These receptors play critical roles in the phagocytosis of *M. tuberculosis* and the triggering of cytokine production [4, 14]. DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin) is a *M. tuberculosis* antigen receptor present primarily on human DCs, although it has also been identified on the alveolar macrophage cell surface of individuals with TB, and therefore may play an important role in propagating an infection [4] (Figure 8).

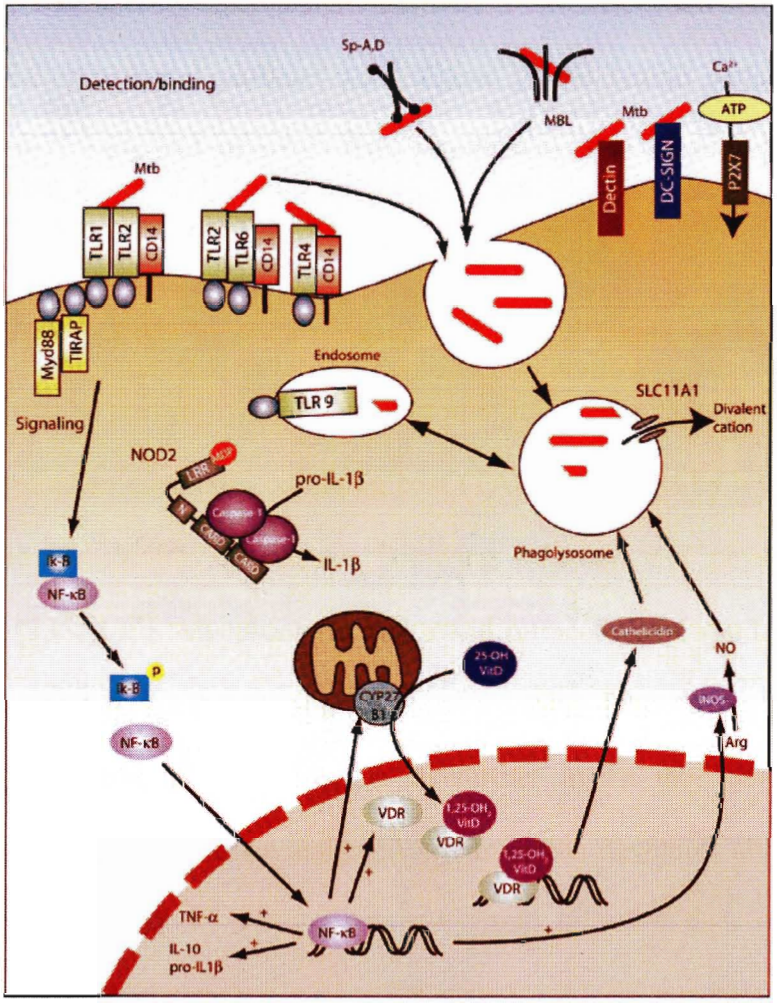


Figure 8: The interaction of *M. tuberculosis* and various macrophage cell receptors in the modulation of the innate immune reaction to infection [4]

Once phagocytosed, the anti-mycobacterial functions of the alveolar macrophages are induced by the fusion of the phagosome and lysosome. The lysosome, with a pH of 4.5-5.0, contains acidic hydrolases. The fusion of this organelle with the phagocytosed *M. tuberculosis* results in bacterial degradation in as little as two hours following entrance into the macrophage [138]. The phagocytosing of *M. tuberculosis* by the macrophage

sends out a signal that attracts macrophages and DCs to the site of infection. DCs engulf or adhere bacteria or bacterial particles and migrate to the lymph nodes, where they present antigens to T cells and stimulate their proliferation and migration to the site of infection [124].

In order to prevent this immune response and remain viable, *M. tuberculosis* has evolved mechanisms to prevent lysosome-phagosome fusion. For example, up-regulating production of tryptophan aspartate-containing coat (TACO) proteins by host cells, which congregate at the phagosomal membrane following infection and physically prevent organelle fusion [138, 139]. Mycobacteria that avoid intracellular degradation multiply, leading to the destruction of the macrophage, and releasing tubercle bacilli that attract more macrophages to the site of infection to further ingest the bacteria. Alveolar macrophages continue to accumulate and mild inflammation develops, although there is little tissue damage [98, 121].

The ligation of *M. tuberculosis* or its products to TLRs causes the macrophage to produce the pro-inflammatory TNF-α, which activates lymphocytes to produce IFN-γ [140]. Together with IFN-γ, macrophages are activated to produce the mycobacteriocidal nitric oxide synthase 2 (NOS2) preventing further replication of *M. tuberculosis* [138, 141]. Patients with active pulmonary TB have been found to have high levels of exhaled NO [138], and NOS2 has also been isolated from the alveolar macrophages of active TB patients [142].

The production of IL-12 is strongly induced by mycobacterial infection and secreted by macrophages, monocytes, neutrophils and T cells. IL-12 stimulates a strong Th1 response to infection by inducing the production of IFN-γ [4]. Significant reductions in bacterial numbers and increases in survival time have been associated with IL-12 treatment of mice [138]. Cells infected by *M. tuberculosis* produce monocyte chemoattractant protein (MCP)-1, which inhibits the production of IL-12 and IFN-γ [143]. Lin and colleagues [144] similarly found that an association between active TB and increased production of MCP-1, as circulating CD14+ immune cells in TB patients have been shown to have increased MCP-1 production, both systemically and locally.

Production of certain anti-inflammatory cytokines also plays a role in reducing T-cell proliferation and function. For example, IL-10 interferes with host defences against *M. tuberculosis* infection by down-regulating the production of TNF-α by macrophages, thereby inhibiting IFN-γ production [138]. Murine models deficient in IL-10 have exhibited lower bacterial burden during early infection [121]. As well, production of IL-6 and TGF-β may play a role in suppressing T cell responses and functions [138].

Antigen presenting cells (APCs), macrophages and DCs, travel to the lymph nodes and present *M. tuberculosis* antigens to naive T cells [98]. As activation of antigen-specific CD4+ cells takes place, large amounts of IFN-γ and TNF-α are produced, assisting the infected macrophage in killing intracellular bacilli [121]. The release of chemokines from macrophages and DCs causes more T cells to activate and aggregate to wall off infection, forming a Th1-mediated granuloma [98]. As depicted in Figure 9, TNF-α plays a major role in the formation and maintenance of the granuloma, influencing

cell migration to the site of the *M. tuberculosis* infection, aggregation and adhesion of cells, and continued activation of macrophages [124, 138]. Neutralization of TNF-α has been shown to cause disorganized granuloma formation and increased bacterial numbers, resulting in 100% mortality in murine models [138]. As well, B cells, DCs, endothelial cells and fibroblasts migrate and form the granuloma [124]. Macrophages further differentiate into epithelioid cells, which form tight linkages with the cell membranes of adjacent cells. As well, multiple macrophages can fuse to form giant cells [145]. In the center of the granuloma, are the infected macrophages releasing enzymes that cause cell lysis and necrosis. Necrosis may occur in an effort to reduce or eliminate *M. tuberculosis*-infected macrophages [98].

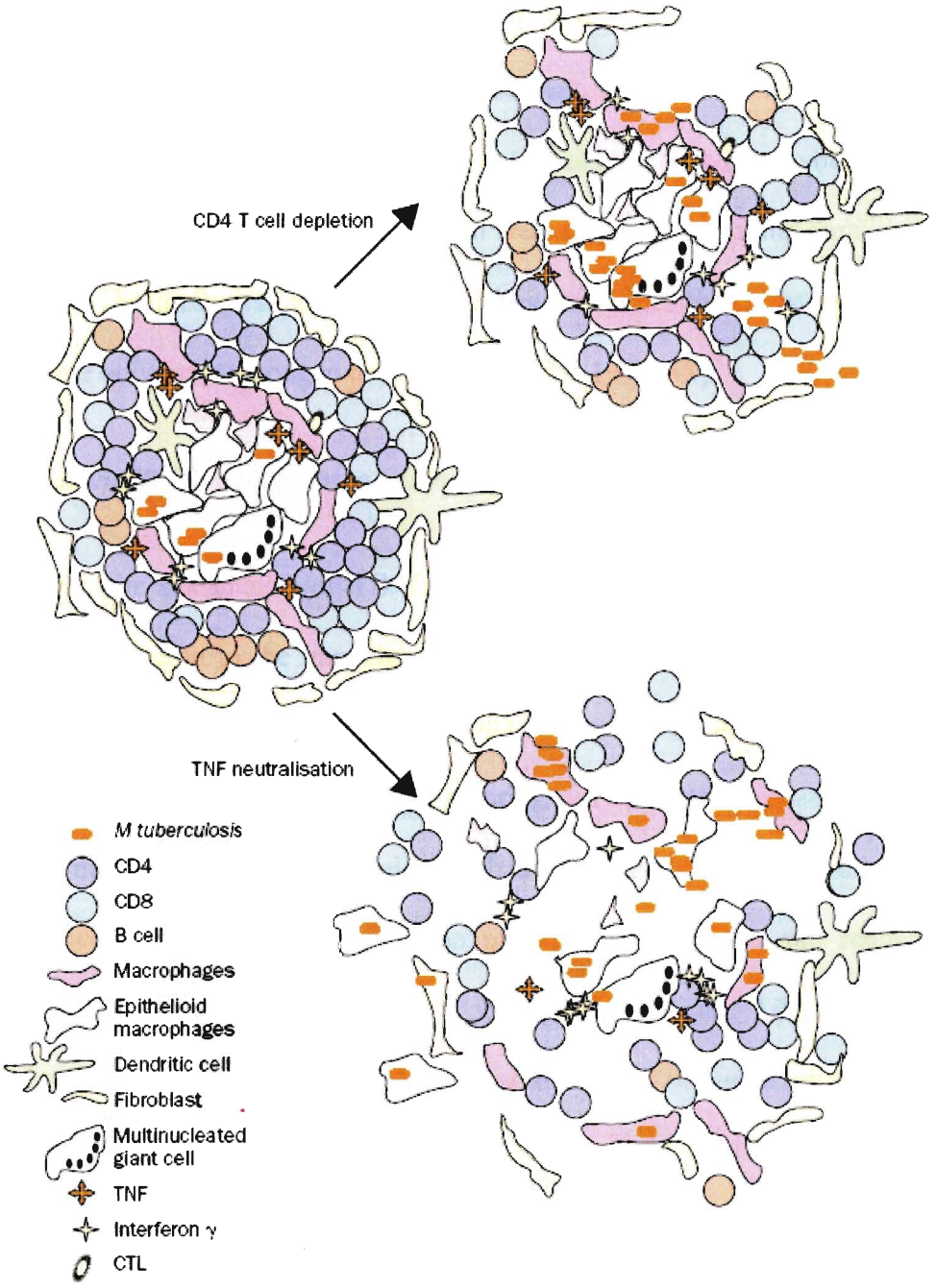


Figure 9: Immune cells and cytokines important to the maintenance of the granuloma during latent tuberculosis infection [124].

2.9 Role of Vitamin D in Immunoregulation of Tuberculosis

The role of vitamin D in combating *M. tuberculosis* infections has been observed and applied since the 1800s, when phototherapy (developed by Niels Ryberg Finsen in 1895 [146]) and the consumption of cod liver oil were observed as having curative effects for TB patients. The biological mechanisms leading to the health improvement of these TB patients have only recently begun to be elucidated [9, 147, 148].

2.9.1 Trends in TB Diagnosis among Immigrants to Non-Endemic TB Regions

Nowadays, the seasonality of TB diagnosis in some high latitude countries has drawn increased attention to the importance of vitamin D in the immune functioning of individuals infected with *M. tuberculosis*. A study in the United Kingdom [149] found that diagnosis of active TB was more common during early summer than winter months, a pattern seen commonly among individuals who had recently immigrated to the UK from the Indian subcontinent. This trend may be partially explained by the reduction in 25(OH)D concentrations that accompanies immigration to the UK. The loss of endogenous vitamin D production is at its greatest at the end of winter, and reactivation and initiation of symptoms, medical attention and diagnosis may be prolonged until summer [149]. Among the population of immigrants to the UK, approximately 809/100,000 people develop active TB within five years of arrival in London [150]. This is also a common trend among immigrants in Canada, who commonly undergo TB reactivation at a higher frequency than their fellow citizens in their countries of origin [122]. Similarly, recent studies in Spain exploring seasonal trends in TB diagnosis

reported a seasonal peak at the end of winter and spring. This type of trend may be explained by several factors such as an increase in indoor exposure to TB during the winter months, long spans of time between the onset of symptoms and diagnosis, and a reduced cutaneous production of vitamin D during the winter [151]. It seems, however, that reduced production of the vitamin is a common denominator in those cases. Vitamin D deficiency may have a synergistic combination with other factors, thus increasing susceptibility to TB reactivation.

Another mechanism linking TB reactivation with vitamin D deficiency is VDR gene polymorphisms that lead to decreased or negligible circulating levels of the vitamin. In a recent study in the UK, TB patients were found to have the lowest 25(OH)D concentrations detected, and were ten times more likely to have undetectable concentrations of circulating 25(OH)D. Although statistically significant associations could not be concluded in this study, *VDR* gene polymorphisms in combination with 25(OH)D deficiency and a reduction in antigenic stimuli may have increased the risk of LTBI reactivation in this immigrant population [152]. Similar studies in the UK reported that serum 25(OH)D concentrations among immigrants from developing countries may decrease by a factor of ten following arrival [15]. Recommending supplements to increase 25(OH)D serum concentrations, therefore, seem like an intuitive solution. However, identifying the target concentration remains elusive. Studies among Asian immigrants to the UK also demonstrated that decreased immunity to TB was not significantly improved by doubling of circulating 25(OH)D by UVB exposure, indicating that higher

concentrations of vitamin D intake may be required in order to achieve significantly increased levels [153].

2.9.2 Latent Tuberculosis Infection and Vitamin D

Decreased circulating 25(OH)D concentrations among immigrants may be partially explained by skin pigmentation. The past 20 years of research have produced studies showing a relationship between individuals with darker skin, vitamin D deficiency and increased susceptibility to *M. tuberculosis* infection. Three recent studies have evaluated the relationship between LTBI and vitamin D. Firstly, a sample population from Guinea-Bissau (12°N) on the western coast of Africa did not show a significant association between LTBI and vitamin D deficiency. It is important to note, however, that this study used TST to determine LTBI status, which may have provided inaccurate results. Due to the low specificity of the TST, many of the individuals determined as LTBI positive may have been, in fact, negative, resulting in an erroneous lack of statistical association between LTBI and serum 25(OH)D concentrations [9]. Secondly, a study among children who had immigrated to the UK reported that 86% of children diagnosed in London with either TB or LTBI had deficient or insufficient 25(OH)D concentrations, as determined by LC-MS/MS [10]. However, this study did not identify if TB/LTBI status was determined by TST or IGRA. Lastly, a retrospective study recently examined the 25(OH)D concentrations of immigrants from sub-Saharan Africa within various health care settings in Australia. Vitamin D deficiency and latent infection with *M. tuberculosis* (as determined by IGRA) were found to be strongly associated in this population [8]. The results of these studies suggest Vitamin D deficiency an important potential risk factor for

TB reactivation, thus continued evaluation and confirmation with more specific testing methods and better study designs are needed.

2.9.3 Genetic Factors

In addition to the mere availability of vitamin D, genetic differences have been proposed as an explanation to the low vitamin D serum levels in certain human groups. In fact, multiple *VDR* gene polymorphisms could have an effect on the host responses to *M. tuberculosis* infection. Time to response to anti-mycobacterial treatment shows a significant association with certain *VDR* gene polymorphisms, suggesting that a change in the structure or activity of VDRs could change the host response to active TB. This genetic variability may partially account for the specific immune responses to TB present in specific ethnic groups [154]. *VDR* gene polymorphisms may identify a possible genetic predisposition to developing TB, although the involvement of specific genotypes is still unknown [155].

Vitamin D deficiency was recently shown to be a significant risk factor for TB reactivation among Gujarati Hindus in the UK. Gujarati Hindus are a primarily vegetarian population of immigrants and the researchers considered that low intake of Vitamin D in their daily diet may have explained the low serum levels of vitamin D. However, within the same population, three *VDR* gene polymorphisms were associated with the severity of vitamin D deficiency and an increased risk of developing active TB. The study concluded that the low concentrations of 25(OH)D in the study population were better explained by genetic polymorphisms rather than an insufficient intake of vitamin D [121].

2.9.4 Innate Immunity Response to Infection

$1,25(OH)_2D$ may carry out its anti-mycobacterial action by regulating the fusion of the phagosome and lysosome, preventing the LAM of *M. tuberculosis* cell wall from disrupting the fusion process [13, 156]. Once endocytosed, *M. tuberculosis* must prevent degradation by inhibiting the fusion of the phagosome and lysosome within the macrophage. As previously mentioned, *M. tuberculosis* avoids phagolysosomal fusion by integrating the host cell TACO proteins with its own cholesterol. However, macrophages treated with $1,25(OH)_2D$ and retinoic acid (RA) have shown a down-regulation of TACO gene expression. This is hypothesized to be mediated by the VDR/RXR complex binding to the TACO gene promoter [139].

Much of the understood interaction between $1,25(OH)_2D$ and *M. tuberculosis* infection involves innate immune mechanisms, as macrophages are the primary host cells for *M. tuberculosis*. Recognition of the bacilli can potentially involve numerous receptors, and trigger multiple pathways within immune cells, as shown in Figure 8. TLR-2 is a pattern-recognition receptor that homodimerizes or heterodimerizes with TLR-1 or TLR-6 on the cell surface of macrophages. The dimerized receptors recognize bacterial antigens [4], thereby activating an anti-mycobacterial response in the macrophage [14]. TLRs interact with MyD88 (myeloid differentiation protein 88), an adapter molecule responsible for initiating intracellular signalling cascade in response to TLR recognition of *M. tuberculosis* (Figure 8) [121]. This signalling pathway leads to the upregulation of VDR and 1-hydroxylase gene expression. 1-hydroxylase converts local 25(OH)D into the biologically active $1,25(OH)_2D$, which then binds to the VDR and enters the macrophage

nucleus, forming a complex with RXR, and binding VDREs in the promoter region of various genes, thereby initiating gene transcription that leads to selective protein synthesis (Figures 8 and 10) [14].

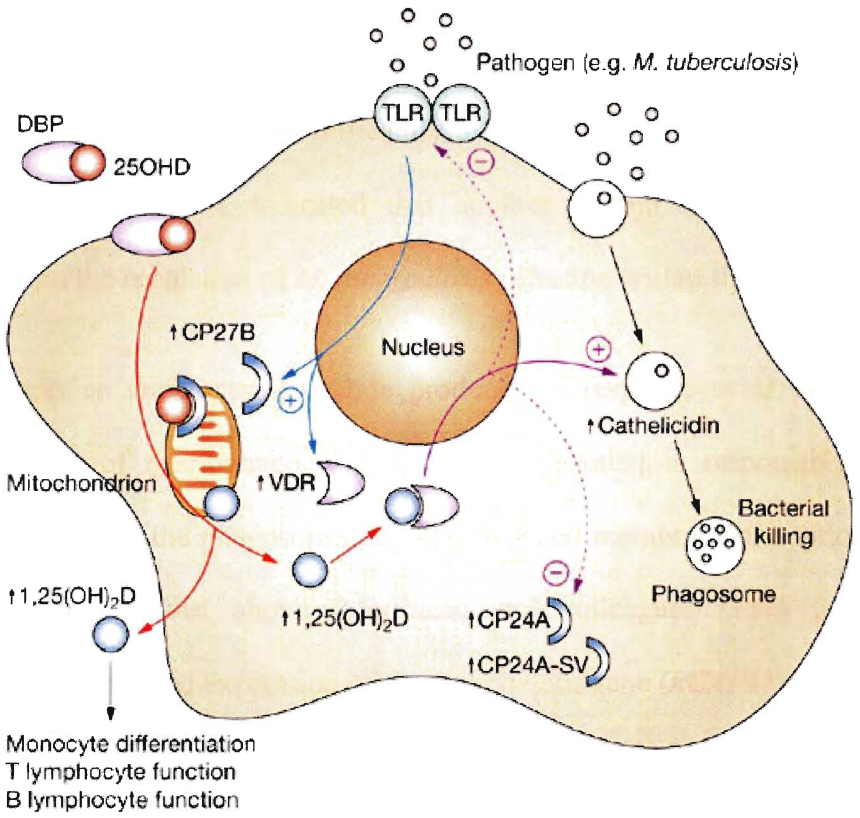


Figure 10: The innate immune response to *M. tuberculosis* infection in the presence of 25(OH)D [97]

Among the proteins up-regulated by $1,25(OH)_2D$, two are of special importance in the regulation of mycobacterial infection in the macrophage: NOS2 and anti-microbial peptide cathelicidin [4, 14, 124, 157]. NOS2 is responsible for the synthesis of NO, toxic to bacteria. Although murine models respond to both acute and chronic *M. tuberculosis* infections with NOS2 production, human macrophages do not produce NOS2 in response

to *M. tuberculosis* stimulation *in vitro*. However, alveolar macrophages of humans with active TB express NOS2, suggesting a species-specificity in reaction to *M. tuberculosis* infection [158]. This finding was supported by Martineau and colleagues [157], who demonstrated that $1,25(OH)_2D$ only moderately up-regulated *NOS2* gene expression in *M. tuberculosis*-stimulated peripheral blood mononuclear cells (PBMC). As well, the inhibition of NO formation did not significantly alter the affect of $1,25(OH)_2D$ on *M. tuberculosis*-infected cells. This indicated that another protein was having a more significant impact on the regulation of *M. tuberculosis* infection within the macrophage.

Cathelicidin is an antibacterial peptide produced in response to *M. tuberculosis* infection (Figure 10) of macrophages [14, 159]. This protein is responsible for *M. tuberculosis* killing within the phagosomes, by bacterial cell membrane disruption [157]. In the same study described above, Martineau and colleagues [157] found that $1,25(OH)_2D$ strongly regulated expression of the cathelicidin gene (*hCAP18*), and that the addition of synthetic cathelicidin reduced *M. tuberculosis* growth in culture by 75.7%. The importance of TLRs and $1,25(OH)_2D$ in inducing expression of this antimicrobial peptide was shown by Liu and colleagues [14], who found that by inhibiting 1-hydroxylase (responsible for synthesizing $1,25(OH)_2D$ in the macrophage), the TLR-induced production of cathelicidin was reduced by 80%, and inhibiting VDR reduced cathelicidin production by more than 80%. As well, *M. tuberculosis* death was reduced by 70%. These findings demonstrate that the TLR-triggered antimicrobial pathway of macrophages is dependent on the production and actions of $1,25(OH)_2D$ and VDR, and

highlights the essential role of $1,25(OH)_2D$ in innate immunity to intracellular bacterial pathogens [14].

In addition to its augmenting effects in the innate immune system by enhancing mycobacterial killing in macrophages, vitamin D metabolites have demonstrated modulatory effects on the adaptive immune system. However, these effects are said to be of immunodepression rather than activation. Experiments show that $1,25(OH)_2D$ interaction with nuclear VDR suppresses the expression of various cytokines involved in the differentiation of naïve T cells (i.e., Th0) into Th1 type cells thereby shifting Th0 differentiation into Th2 cells. Th1 cells produce pro-inflammatory cytokines (such as IFN-γ, TNF-α, IL-12, IL-8, IL-6 and IL-10) and a reduction in their production has anti-inflammatory properties [14, 160]. An anti-inflammatory effect may actually have a protective role in infectious diseases as it is widely known that uncontrolled inflammation may lead to not only more tissue damage but to pathogen spread [12]. Controlling bacterial spread is of particular importance in TB.

Since vitamin D production is enhanced by UVB rays during sun exposure, the anti-inflammatory action of vitamin D is considered an evolutionary protective mechanism [161]. This immunosuppresion is compensated though, by the increased production of the antibacterial cathelicidin peptide [12, 161]. The protective role of vitamin D in TB has been demonstrated repeatedly. Numerous studies have shown deficient levels of the hormone being strongly associated with active disease [6, 147, 162]. Furthermore, other studies have also shown that ethnic groups considered at high risk for TB have generally lower levels of vitamin D and cathelicidin expression, such as the study by Wang and

colleagues [100] who established this difference among African Americans when compared to Whites in the United States. The fact that those identified as high risk are dark-skinned populations is interesting. Social determinants aside, it may very well be that the interference of dark skin in vitamin D synthesis is playing a significant role among these populations.

With all the increasing evidence that vitamin D deficiency is a major a risk factor for TB reactivation, it can be hypothesized that the vitamin somehow plays a vital role in the immune maintenance and integrity of the granuloma, thus preventing latent infection from becoming active. The WHO data affirms that one third of the world population harbours latent TB infection and that the majority of the 8-9 million TB clinical cases per year are due to reactivation of a latent infection [116]. Preventing reactivation is paramount to achieving the MDG 6 by 2015 [163]. With this in mind the scientific community is currently engaged in the creation of new vaccines to prevent TB infection and new and better drugs to treat and cure it. At the same time, alternative approaches including immunomodulators are being investigated. Among these, the potential of vitamin D as a low-cost and easily manageable risk factor for developing active TB has been received with much optimism among the scientific and clinical community [157]. The aim of the present study is to contribute to the understanding of the potential role of vitamin D in latent TB infection in a population from a medium-TB burden country.

CHAPTER THREE: METHODOLOGY

The purpose of this study was to determine frequency of vitamin D deficiency as a risk factor for latent TB reactivation among Mexican migrant workers in the Niagara Region. This study was an extension of a larger study that was initiated in March 2007 as the graduate project of Angela Duarte (supervised by Dr. A. Sanchez) in collaboration with the University of Puebla in Mexico, entitled "Preliminary Epidemiological Study of Latent Tuberculosis in Mexican Agricultural Workers in the Niagara Region, Canada", and funded by the CIHR. Duarte's study set out to assess the prevalence of LTBI, and associated risk factors for infection, in a population of Mexican agricultural workers in the Niagara Region of Ontario, Canada.

Various farms in the Niagara Region were contacted before workers could be approached for participation. Within each farm agreeing to participate, individuals providing free and informed consent were enrolled. Participation included completion of a questionnaire, TST and extraction of a blood sample for the IGRA. Questionnaires contained questions regarding details of enrolment, living conditions in Canada, socio-demographic data in Mexico, and TB knowledge and history. Questionnaires were distributed and explained in Spanish. LTBI was assessed by TST as a screening test, and an IGRA was performed as a confirmatory test. TSTs (Tubersol®, Avantis Pasteur Limited) were performed by registered nurses, and trained phlebotomists took the blood samples for the IGRA (QuantiFERON-TB Gold In-Tube®, Cellestis Limited, Australia) (see Appendix II for QuantiFERON-TB Gold In-Tube® technique). Blood samples were consistently taken in the evening, following work hours during August 2007. This

preliminary study on latent TB conformed to the research ethics standards outlined in the Tri-Council Policy Statement [164] and received Research Ethics Board approval from Brock University, File #DUARTE06-288. Participants gave consent and provided a blood sample to be tested for levels of vitamin D, which was analyzed using a code number, centrifuged and the serum stored at -20°C for use at a later date. These samples were tested for circulating 25(OH)D concentrations in the present study.

3.1 Research Objectives

3.1.1 General Research Objective

To determine the frequency of vitamin D deficiency, a risk factor for LTBI reactivation, among healthy individuals with known status for latent TB infection.

3.1.2 Specific Research Objectives

Objective 1. Using a widely accepted radioimmunoassay, determine the serum 25(OH)D concentrations among healthy individuals from a TB-endemic country, and establish the proportion of individuals with sufficient, insufficient and deficient vitamin D status.

Objective 2. Determine if a statistically significant difference existed between the 25(OH)D concentrations of males and females, as well as between age categories (defined in a later section). As well, determine if a statistically significant difference existed between the vitamin D status (sufficiency, insufficiency and deficiency) of males and females, as well as between age categories.

Objective 3. Analyze what variables are independent predictors of 25(OH)D concentrations and vitamin D status, including age, sex, number of years enrolled in the Seasonal Agricultural Workers Program (SAWP), number of months in Canada during the present season, alcohol consumption, latitude of Mexican state of origin, and completion of primary school.

Objective 4. Establish 25(OH)D concentrations and vitamin D status of individuals in the LTBI-negative and LTBI-positive groups, and determine if a significant difference existed between the 25(OH)D concentrations and vitamin D status between LTBI-negative and LTBI-positive groups.

Objective 5. Determine if serum 25(OH)D concentrations were statistically correlated with interferon-gamma concentrations previously determined, in the overall sample and among LTBI-positive participants.

3.2 Research Design

This study was a cross-sectional analysis of circulating 25(OH)D concentrations among a convenience sample of 92 Mexican workers within the Niagara Region's agricultural industry.

Circulating concentrations of 25(OH)D were quantified using a radioimmunoassay (RIA), allowing for the determination of vitamin D sufficiency, insufficiency and deficiency based on previously determined cut-points. These allowed for the determination of the point prevalence of vitamin D deficiency and insufficiency.

The vitamin D analysis will be performed in a blind fashion with respect to the LTBI status of individual participants.

3.3 Study Funding

The present study received funding through a Brock University Advancement Fund, awarded to Dr. Paul LeBlanc as a Principal investigator and Dr. Ana Sanchez as a co-investigator. Partial funding was also obtained from the Canadian Institutes of Health Research (CIHR).

3.4 Ethical Considerations

Informed consent was obtained from all research participants in the summer of 2007, as was approval from Brock University's Research Ethics Board. Consent forms were distributed and signed by participants following the explanation of the nature of the research project, the study's procedures, potential risks and benefits associated with the study, procedures by which confidentiality will be maintained and assurance of the voluntary nature of participants' involvement. A modification to file number DUARTE06-288 adding Dr. LeBlanc and Timna Merion as researchers was submitted and accepted by the REB of Brock University, allowing access to anonymous data.

The present study involved the manipulation of previously collected serum samples and anonymized questionnaire data, and therefore presented no physical or other risks to the participants. Continued confidentiality of participants' data was ensured.

3.5 Biosafety and Radiation Safety

Biosafety and radiation safety training sessions were carried out by Brock University's Environment, Health and Safety Office before beginning laboratory procedures. This was followed by on-site radiation training by Dr. P. LeBlanc in his laboratory, which consisted of proper handling and storage of radioactive materials, use of the gamma radiation counter, record keeping and clean-up procedures.

Initial blood and serum sample manipulation was performed in Dr. A. Sanchez's laboratory, accredited Biosafety Level II. Radioimmunoassay techniques were performed in a designated radioactivity bench at Dr. Paul LeBlanc's laboratory, by Timna Merion, under the supervision of thesis advisors, Dr. A. Sanchez and Dr. P. LeBlanc. Lab coats and disposable gloves were worn while handling biological and radioactive materials. All radioactive materials were carried out on a designated radiation lab bench.

3.6 Laboratory Methodology: 25-Hydroxyvitamin D ^{125}I Radioimmunoassay

3.6.1 Background and Principle of the Assay

After entering circulation, vitamin D is metabolized into many forms, primarily 25(OH)D. Since this is the most abundant form in which vitamin D is stored in the body, this is the metabolite for which this assay is testing [165]. This is the metabolite most commonly used to determine vitamin D status [21, 58, 166-170].

The DiaSorin 25(OH)D RIA procedure consists of two steps. First, 25(OH)D and other hydroxylated metabolites are rapidly extracted from the serum samples. Second, the

samples are assayed using an equilibrium RIA method, which uses an antibody specific to 25(OH)D[165].

3.6.2 Assay Technique

3.6.2.1 Extraction Procedure

Samples and standards were aliquoted in duplicates. 12 X 75 mm disposable glass tubes were set up and labelled for calibrators, controls and research participants samples. 500 µL of acetonitrile were added to each tube using a pipette. 50 µL of the calibrator, control or patient were added to the respective tube by placing the pipette tip below the surface of the acetonitrile and slowly adding the contents. Once all samples were completed, each tube was vortexed for 10 seconds. The tubes were all centrifuged at 1200 X g ($g = (1118 \times 10^{-8})$ (radius in cm) $(rpm)^2$) for 10 minutes at 20-25°C.

25 µL aliquots of supernatant were pipetted in duplicates into labelled 12 X 75 mm disposable glass tubes. Supernatants were assayed as indicated by the Assay Procedure, outlined below.

3.6.2.2 Assay Procedure

This procedure was carried out on a designated radiation laboratory bench, and all pipettes used were labelled as radioactive. All reagents and samples were equilibrated to room temperature (up to a temperature of 25°C). Disposable glass tubes were labelled and set up in duplicates in the order of "Total Counts", "NSB", "Calibrator 0" to "Calibrator 5", "Controls" and "Unknown Samples".

Reagents were added as follows:

1. Total Count tubes:

    - 50 µL of ^{125}I 25(OH)D tracer
    - 1.0 mL of NSB/Addition buffer

2. Non-specific binding tubes (NSB):

    - 25 µL of Extracted 0 calibrator
    - 50 µL of ^{125}I 25(OH)D tracer
    - 1.0 mL of NSB/Addition buffer

3. Calibrators, controls, and unknown samples:

    - 25 µL of calibrator, control, or unknown sample (extracted)
    - 50 µL of ^{125}I 25(OH)D tracer
    - 1.0 mL of 25(OH)D antiserum

Each tube was gently vortexed to avoid foaming, and incubated for 90 (± 10) minutes at 20-25°C.

DAG precipitating complex was poured into a beaker and stirred with a stir bar before and during use. 500 µL DAG was added to all tubes, except the "Total Count" tubes. All tubes were mixed well and incubated for 20-25 minutes at 20-25°C.

500 µL of NSB/Addition buffer was added to all tubes, except the "Total Count" tubes, and vortexed gently to avoid splashing (although the tubes were mixed well). All tubes were centrifuged for 20 minutes at 20-25°C at 1800 X g, except the "Total Count" tubes.

Supernatants were decanted (except for the "Total Count" tubes) by inverting each tube into an appropriate waste container, and then each tube was inverted and placed in a rack over absorbent paper for 2-3 minutes. Each tube was gently blotted to remove all liquid.

3.6.2.3 Radiation Measurement

A Gamma scintillation counter (Wallac Wizard 1470 Automatic Gamma Counter, model #1056 1349) was used to quantify the concentrations of 25(OH)D in the serum samples. All the tubes were placed in a gamma scintillation counter in groups of ten, and counted for at least 1 minute. Results of the standards were plotted to construct a standard curve and printed.

3.7 Cut Points Selected to Define Vitamin D Levels

Based on the literature [61-67, 171], cut points will be defined as follows:

Vitamin D sufficiency: $\geq$80 nmol/L

Vitamin D insufficiency: <80 nmol/L, >37.5 nmol/L

Vitamin D deficiency: $\leq$37.5 nmol/L

3.8 Determination of LTBI – Summary of the Technique and Interpretation

LTBI was determined by the interferon-gamma release assay (IGRA), QuantiFERON-TB Gold In-Tube® (Cellestis Limited), which incorporates proteins early

secretory antigenic target (ESAT-6), culture filtrate antigen (CFP-10) and TB7.7. This test quantifies the IFN-γ released by T lymphocytes in the whole heparinised blood of individuals, following stimulation with the *M. tuberculosis* proteins listed above. The antigens used in this test are not present in any strain of BCG, and therefore are not likely to produce a reaction in individuals who have been immunized, but are uninfected. This test is therefore an indicator of LTBI and a supporting test for the diagnosis of active TB[172].

First, whole blood was collected directly into three specialized tubes, including a Nil Control tube, TB Antigen tube (containing the protein cocktail), and a Mitogen Control tube, and incubated at 37°C. Secondly, the tubes were centrifuged and the plasma was removed. An ELISA was used to quantify the IFN-γ response. The antigen tube was considered to have a positive IFN-γ response when it is significantly higher than the IFN-γ response of the Nil tube[172].

3.9 Predictors of Vitamin D Status

Factors identified in the literature as having an association with circulating 25(OH)D concentrations have include age [34, 53, 167, 173], skin pigmentation [152, 153, 174], diet [175], sun protection [49], latitude [153, 174] and season [167, 176]. There has also been some analysis of the association between vitamin D deficiency and alcohol consumption [177] and education level [9, 178, 179]. The current study included the following independent predictors of vitamin D deficiency and 25(OH)D concentration:

- Age
- Sex
- Number of years with in SAWP
- Length of stay in Canada
- Latitude of Mexican state of origin
- Levels of completed education
- Alcohol consumption

Data for these variables were taken from the questionnaire, with exception of two, number of months in Canada and latitude of Mexican state of origin, which were calculated from data provided in the participants' questionnaires. Number of months in Canada was calculated from month of arrival to month at which blood samples were taken (month of arrival was provided). Participants provided their state of origin on the questionnaire, from which the approximate latitude was determined [180] from the state of origin provided by participants in the questionnaire. Age remained as a continuous variable, but was also described in terms of categories for the purpose of showing age distributions. Age was categorized into 20-29, 30-39, 40-49 and ≥50 years. Selection of potential predictors of 25(OH)D concentration was limited by the questionnaire content. Therefore risk factors such as skin pigmentation, diet and sun protection measures could not be included.

3.10 Data Analysis

Objective 1. Means and standard deviations (SD) were used to describe the serum 25(OH)D concentrations of the overall population, as well stratified by sex and age

categories. Frequencies and percentages described the vitamin D status (deficiency, insufficiency and sufficiency) of the overall population, as well stratified by sex and age.

Objective 2. A t-test was used to determine if there was a significant difference between the 25(OH)D concentrations of males and females, and an ANOVA was used to determine if a significant difference existed between age categories. Fisher's exact tests were used to determine if vitamin D status significantly differed between sex or age categories.

Objective 3. In order to assess which variables were independent predictors of 25(OH)D concentration and vitamin D status, a multiple linear regression (MLR) and logistic regression were performed, respectively. Backward selection was used to determine inclusion in the regression models, in which variables with a p value ≥ 0.20 were eliminated from the models [181]. Vitamin D deficiency was the reference group in the logistic regression.

Objective 4. Descriptive statistics of the serum 25(OH)D concentrations stratified by LTBI status was presented as means and standard deviations, and vitamin D status stratified by LTBI status was presented as frequencies and percentages. A t-test was used to determine if 25(OH)D concentration was significantly different between LTBI-negative and LTBI-positive participants. A Fisher's exact test was used to determine if vitamin D status significantly differed between LTBI-negative and LTBI-negative participants.

Objective 5. A Spearman correlation was used to assess if there was a significant correlation between 25(OH)D and IFN-γ concentrations within the overall population and

within the LTBI-positive group. The strength of the association was determined by the correlation coefficient.

Statistical analysis of the data was carried out using the software package SAS version 9.1 (SAS Institute, Cary NC, USA). Significance was determined by 95% confidence intervals and a p value of $p<0.05$ for all analyses. Assumptions for the statistical analyses were checked and met. Serum 25(OH)D concentrations were log transformed in order to meet the assumption of normal distribution for linear regression, and back-transformed by taking the antilog before interpretation of the results.

CHAPTER 4: RESULTS

Of the 92 serum samples collected by Duarte from Mexican agricultural workers in the Niagara Region during August of 2007, 87 were included in the present study. The samples had been stored at -20°C in plastic Eppendorf tubes during the interim, and assayed for concentrations of the vitamin D metabolite 25(OH)D between January and February of 2009 using a 25(OH)D ^{125}I radioimmunoassay (RIA) kit purchased from DiaSorin®. The results of five serum samples were not included in the present study. Three of these were not included because they contained an insufficient volume of serum for the assay to be carried out (50μL was required). The results of the remaining two participants were not included because their coefficient of variation (CV) percentages did not meet predetermined cut-offs of 10%. Repeat analyses of these two samples could not be carried out as planned due to an insufficient volume of serum remaining.

Training and preparation for use of the DiaSorin® 25(OH)D RIA to assay the serum samples took place regularly over a period of three months. Training consisted of three stages before samples from the present study were handled and assayed. Firstly, training workshops for both radiation and biosafety were completed at Brock University. Secondly, samples of the researchers and other volunteers from Brock University were collected and assayed. This exercise was used to gain experience and comfort with the assay procedure, and address potentially problematic steps in the procedural process. Thirdly, 46 serum anonymized samples from Puebla, Mexico, collected during the study led by Pezzat and collaborators in June 2008, were assayed in triplicate using the RIA kits. These samples were collected as part of a complementary study, and results were

sent back to the Mexican researchers in January 2009 for their decoding and interpretation. Consistently low CV percentages (<10%) for intra-assay variability among the Puebla specimens, which were all tested in triplicate, conferred confidence that the use of duplicate samples, as recommended by the manufacturer, may be used thereafter.

25(OH)D concentrations were measured with a Gamma Radiation Counter and output data was produced in count per minute (CPM). The CPM of the calibrators, total counts and NSB buffer were used to generate a calibration curve in Microsoft Excel® using the procedure and formulas provided by DiaSorin®. A new set of calibrators were assayed and new calibration curve produced for each set of samples. The 25(OH)D concentrations were calculated from the calibration curve equation, yielding a concentration in ng/mL, which was subsequently converted to nmol/L by multiplying by a factor of 2.496.

$$\frac{\text{nmol 25(OH)D}}{\text{L}} = \frac{2.496 \text{ x ng 25(OH)D}}{\text{mL}}$$

Demographic characteristics and tuberculosis-related data were obtained from the surveys which were carried out as part of the Duarte study. All variables were entered into SAS Version 9.1 for statistical analysis.

4.1 Description of the Research Participants

As previously stated, 87 (94.6%) of the original 92 study participants were included in the present study, including 68 (78.2%) males and 19 (21.8%) females. The average age of the participants was 38.0 (± 9.14) years, with ages ranging from 22-65 years.

Among male participants, the average age was 39.1 (± 9.27) years, ranging from 22-65 years, and the average female age was younger at 34.1 (± 7.65) years, ranging from 24-49 years. As depicted in Table 4, 78.2% of the sample was made up of male participants, while 21.8% was made up of females. The average time of participation in the SAWP program was 6.3 years (±5.68) (6.8 years (±5.8) for males and 4.4 years (±4.0) for females). The latitude of origin for each participant varied between 16°-25°N, majority of whom originate from approximately 19°N (61%) and 21°N (15%). Other demographic characteristics such as age distributions, number of years of SAWP enrolment, Mexican state of origin, type of housing in Mexico, level of completed education and the number of months in Canada are presented below in Table 4. Fourteen out of the 87 (16.1%) participants were LTBI positive, as determined by the QuantiFERON-TB Gold In-tube® IFNγ release assay.

Table 4: Demographic characteristics from a sample of 87 Mexican agricultural workers in the Niagara Region, stratified by sex

Characteristic		Frequency (%)		
		Males	Females	Total
Age (years)	20 – 29	10 (14.7)	7 (36.8)	17 (19.5)
	30 – 39	29 (42.7)	6 (31.6)	35 (40.2)
	40 – 49	20 (29.4)	6 (31.6)	26 (29.9)
	≥50	9 (13.2)	0 (0)	9 (10.3)
Number of years enrolled with SAWP	1-5	37 (54.4)	12 (63.2)	49 (56.3)
	6-10	17 (25.0)	6 (31.6)	23 (26.4)
	11-15	4 (5.9)	1 (5.3)	5 (5.8)
	16-20	7 (10.3)	0 (0)	7 (8.1)
	21-25	3 (4.4)	0 (0)	3 (3.5)
Type of housing in Mexico[†]	Type I	48 (71.6)	13 (72.2)	61 (71.8)
	Type II	15 (22.4)	3 (16.7)	18 (21.2)
	Type III	3 (4.5)	2 (11.1)	5 (5.9)
	Type IV	1 (1.5)	0 (0)	1 (1.2)
Level of education completed	None	10 (14.7)	1 (5.3)	11 (12.6)
	Primary	31 (45.6)	4 (21.1)	35 (40.7)
	Secondary	24 (35.3)	10 (52.6)	34 (39.5)
	Technical	3 (4.4)	3 (15.8)	6 (7.0)
	Other	0 (0)	1 (5.3)	1 (1.2)
Length of stay in Canada (months)	2	2 (3.0)	6 (31.6)	8 (9.3)
	3	0 (0)	0 (0)	0 (0)
	4	19 (28.4)	9 (47.4)	28 (32.6)
	5	37 (55.2)	3 (15.8)	40 (46.5)
	6	9 (13.4)	1 (5.3)	10 (11.6)

[†] Type of housing is classified according to the presence of certain household amenities, including indoor potable water, indoor flushing toilet, indoor electricity, and cement or tiled floors. Type I: All four characteristics are present; Type II: Any three characteristics are present; Type III: Any two characteristics are present; Type IV: One characteristic is present

4.2 Vitamin D Status

4.2.1 25(OH)D Concentrations in the Study Population

The mean 25(OH)D concentration of all participants was 74.9 nmol/L (±20.1), ranging 31.9-149.7 nmol/L. Seven participants had concentrations ≥100 nmol/L. Mean 25(OH)D concentration in this population did not differ by sex or age classifications, as shown in table 5.

Table 5: 25(OH)D concentration of Mexican agricultural workers classified by sex and age category (n=87)

	25(OH)D Concentration (nmol/L) (± SD)
Sex	
Male	76.6 (±20.8)
Female	68.8 (±16.3)
$p = 0.16$	
Age category (years)	
20-29	76.0 (±3.57)
30-39	68.4 (±18.61)
40-49	77.8 (±24.79)
≥50	78.3 (±14.48)
$p = 0.26$	

Normality of the 25(OH)D concentration distribution was tested using the Anderson-Darling test for normality, which yielded a p value of 0.005, indicating that 25(OH)D concentrations were not normally distributed. In order to achieve a normal distribution, the concentrations were log transformed prior to analysis, for which the

Anderson-Darling test yielded a p value of 0.08, indicating normal distribution. In order to satisfy the assumption of normal distribution required for Student's t-test and linear regressions, the log of the 25(OH)D concentrations was utilized for analysis, and then converted back from the log value to the antilog. A Student's t-test was performed in order to find if a significant difference existed between the mean 25(OH)D concentrations of males and females. The t-test indicated that there was no significant difference between the 25(OH)D concentrations of males and females (p=0.16). Using an ANOVA it was determined that 25(OH)D concentrations did not differ significantly among the age categories (p=0.26).

4.2.2 Categorizing the Vitamin D Status of the Study Participants

As previously stated, the 25(OH)D concentrations were categorized into sufficiency (≥80 nmol/L), insufficiency (<80 nmol/L) and deficiency (≤37.5 nmol/L). Table 6 shows the frequency distributions of each of these categories.

Table 6: Vitamin D status of Mexican agricultural workers classified as deficiency, insufficiency and deficiency (n=87)

Vitamin D Status	Frequency (%)
Deficiency (≤37.5 nmol/L)	2 (2.3)
Insufficiency (<80 nmol/L)	59 (67.8)
Sufficiency (≥80 nmol/L)	26 (29.9)

Since the deficiency category only contained two participants, it was merged with the sufficiency category to create a dichotomous vitamin D status variable. Therefore, 70.1% of participants were classified as having as an insufficient 25(OH)D concentration

(<80 nmol/L) and 29.9% of participants were classified as having a sufficient concentration (≥80 nmol/L).

Table 7 shows the frequency of vitamin D insufficiency and sufficiency in the population, stratified by sex. One of the cells had a frequency of less than 5, which does not meet the requirement of a Chi-squared test. Instead, a Fisher's exact test was performed in order to evaluate if a statistical association existed between vitamin D status (i.e. sufficiency versus insufficiency) and sex. The *p* value computed by the Fisher's exact test was p=0.16, indicating that there was no significant relationship between vitamin D status and sex in this study population.

Table 7: Vitamin D insufficiency and sufficiency of Mexican agricultural workers, stratified by sex (n=87)

Vitamin D Status	Frequency (%)	
	Male	Female
Insufficient (<80 nmol/L)	45 (66.2)	16 (84.2)
Sufficient (≥80 nmol/L)	23 (33.8)	3 (15.8)
$p = 0.16$		

Table 8 shows vitamin D status stratified by age categories. A Fisher's exact test was used to evaluate the relationship between the two variables. It was determined that there was no significant relationship between vitamin D status and age (p=0.36).

Table 8: Vitamin D status of Mexican agricultural workers stratified by age (years)

Vitamin D status	Frequency (%) by age category (year)			
	20-29	30-39	40-49	≥50
Insufficiency	8 (9.2)	20 (23.0)	23 (26.4)	10 (11.5)
Sufficiency	1 (1.2)	6 (6.9)	12 (13.8)	7 (8.1)
$p = 0.36$				

4.3 Predicting 25(OH)D Concentration and Vitamin D Status

4.3.1 Identifying Predictors of Vitamin D Status

Logistic regression was carried out in order to assess the predictive effect of specific factors on vitamin D status (sufficiency/insufficiency). Potential predictor variables were taken from published peer-reviewed literature, including sex, number of years enrolled with SAWP, length of stay in Canada (current growing season), latitude of Mexican state of origin, education level completed, and alcohol consumption. Variables were removed from the model by backward elimination selection [181], and inclusion was determined by a significance value of $p<0.20$ [181]. An effect of collinearity was found between age and number of years enrolled in the SAWP, and therefore the inclusion of number of years of enrolment acts as a proxy for age. Table 9 shows the results of the analysis, including the odds ratios for sex, number of years in the SAWP, length of stay in Canada (current growing season) and completion of primary school. A significant association was not found for latitude of Mexican state of origin and alcohol consumption.

Table 9: Covariates of logistic regression predicting vitamin D status of Mexican agricultural workers[#]

Variable	Odds Ratio (95% CI)	*p* value
Sex (female)[†]	0.06 (0.01, 0.37)	0.01
Number of years enrolled with the SAWP	0.86 (0.76, 0.98)	0.02
Length of stay in Canada (months)	0.33 (0.16, 0.68)	0.003
Completion of primary school[‡]	2.60 (0.83, 8.17)	0.10

[†] Female sex was compared to male sex
[‡] Completion of primary school was compared to no schooling completion
[#] Eliminated variables (p>0.20): latitude of Mexican state of origin, alcohol consumption

Females were 94% more likely to be insufficient than men. As well, with each year increase in age, individuals were 14% more likely to have insufficient concentrations of 25(OH)D. Similarly, within each growing season, for each additional month in Canada, individuals are 67% more likely to have an insufficient vitamin D status. The OR of primary school completion was not significant in a 95% confidence interval. In order of elimination from the model, alcohol consumption and latitude of the state of origin were not found to be significant independent predictors of vitamin D status, with p values of 0.42 and 0.30, respectively.

4.3.2 Identifying Predictors of 25(OH)D Concentrations

In order to assess which demographic factors are independent predictors of 25(OH)D concentration, a multiple linear regression (MLR) was performed. Predictor variables were included based on the current literature, and removed from the model in a backward selection procedure [181]. Variables with a p-value $\geq$0.20 were eliminated from the model [181]. The number of years enrolled with the SAWP was used as a proxy for age, due to an effect of collinearity between the two variables. Potential predictor

variables analyzed included: sex, number of years enrolled with the SAWP, length of stay in Canada during the current growing season, latitude of Mexican state of origin, education level completed, and alcohol consumption. Table 10 shows the results of the MLR for each of demographic factors that were included in the model, which included sex, number of years enrolled in the SAWP, length of stay in Canada (current growing season) and education completed. A significant association was not found for alcohol consumption or latitude of Mexican state of origin. Completion of secondary and technical schools did not yield significant associations; therefore completion of primary school was compared to no schooling completion.

Table 10: Covariates of the multiple linear regression predicting 25(OH)D concentrations of Mexican agricultural workers[#]

Covariate	Coefficient (95% CI)	*p* value
Sex (female)[†]	-1.56 (-2.19, -1.12)	0.01
Number of years enrolled in SAWP	-1.03 (-1.05, -1.00)	0.02
Length of stay in Canada (months)	-1.21 (-1.38, -1.06)	0.007
Completion of primary school[‡]	1.18 (-1.09, 1.52)	0.195

[†] Female sex was compared to male sex
[‡] Completion of primary school was compared to no schooling completion
[#] Eliminated variables (p>0.20): latitude of Mexican state of origin, alcohol consumption

The 25(OH)D concentration of females was 1.56 nmol/L less than males. Each additional year enrolled in SAWP resulted in a 1.03 nmol/L reduction in 25(OH)D concentration. As well, for every additional month in Canada (in the current growing season) the concentration of 25(OH)D was reduced by 1.21 nmol/L. Primary school completion was not a significant predictor of 25(OH)D concentration within a 95%

confidence interval. The R^2 of the model was 0.1949, and therefore 19.49% of the variance in 25(OH)D concentration can be predicted by the model. In order of elimination, excluded variable and corresponding p-values were latitude of Mexican state of origin (p=0.77) and alcohol consumption (p=0.67).

4.4 Relationship between Vitamin D Status and LTBI Status

Of the 87 research participants, 14 (16.1%) were determined LTBI positive by the QFT-G IT assay (Duarte et al, 2009; submitted for publication). A Fisher's exact test was performed, and determined that there was no significant relationship between vitamin D status and LTBI status in this study population (p=0.54) (Table 11). One of the participants had an indeterminate LTBI status, and was not included in this analysis.

Table 11: Vitamin D insufficiency and sufficiency of Mexican agricultural workers, stratified by LTBI status (n=86) †

Vitamin D Status	Frequency (%)	
	LTBI positive (n=14)	LTBI negative (n=72)
Insufficiency	11 (78.6)	49 (68.1)
Sufficiency	3 (21.4)	23 (31.9)
$p = 0.54$		

† One person not included due to an indeterminate LTBI status

Within the LTBI negative group, the mean 25(OH)D concentration was 75.1 nmol/L (±21.3). The mean 25(OH)D concentration for the LTBI positive group was 74.1 nmol/L (±12.3). A Student's t-test determined that no significant difference existed between the 25(OH)D concentrations of LTBI positive and negative individuals (p=0.89).

4.5 Association between 25(OH)D Concentration and IFN-γ Concentration

To determine if a linear relationship existed between concentrations of 25(OH)D and IFN-γ, a correlation analysis was performed (n=87). Due to the fact that neither variable was normally distributed, a Spearman correlation was carried out. The correlation coefficient obtained was 0.14 (p=0.19), and therefore not statistically significant. A Spearman correlation was also carried out between concentrations of 25(OH)D and IFN-γ of the LTBI positive participants (n=14). This yielded a correlation coefficient of 0.53 (p<0.05), indicating a moderate correlation between the 25(OH)D and IFN-γ concentrations among individuals who are positive for LTBI (Figure 11).

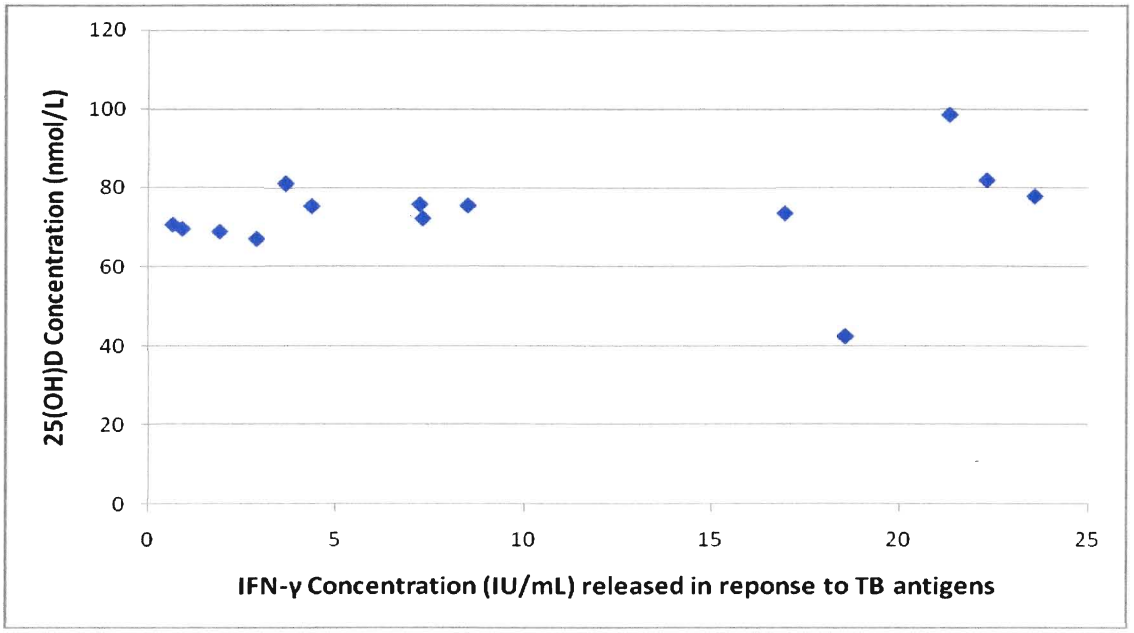


Figure 11: Spearman correlation between 25(OH)D concentrations and IFN-γ release by effector T cells of LTBI-positive participants

CHAPTER 5: DISCUSSION

The present study has focused on determining the vitamin D status of Mexican migrant workers in the Niagara Region, with the particular aim of evaluating the relationship with LTBI. The WHO's estimate that one third of the world is latently infected with *M. tuberculosis* [116], and research suggesting vitamin D deficiency may lead to LTBI reactivation, highlight the importance of defining the relationship between these two conditions.

The role of vitamin D metabolites in the regulation of calcium homeostasis has long been acknowledged. These metabolites, 25(OH)D and $1,25(OH)_2D$, respectively function as the primary storage and biologically active form of vitamin D [59]. More recently, production of $1,25(OH)_2D$ had been shown to be more ubiquitously distributed throughout the body, and involved in the upregulation of the gene coding for cathelicidin, an anti-mycobacterial protein [14].

Many LTBI reactivation studies have been carried out among foreign-born populations from TB-endemic, tropical or subtropical regions that have re-located in developed regions at higher latitudes, where sun exposure is reduced, leading to reduced production of vitamin D in the skin. In Canada and the UK, since the onset of TB symptoms and diagnosis take place most often within the first five years following immigration, it has been theorized that vitamin D deficiency due to reduced sun exposure may play an immunoregulatory role in reactivation of LTBI [122, 150]. Although a definitive association has not been established for the relationship between vitamin D

deficiency and LTBI reactivation, it remains a potential risk factor for reactivation and progression to active TB. Further study of vitamin D status may contribute to the understanding of LTBI in migrant populations.

5.1 Characteristics of Research Participants

Mexican agricultural workers have been coming to work in Canada with the Seasonal Agricultural Work Program (SAWP) since 1974 [182], with women entering the program in 1989 [183]. Employment criteria and recruitment processes require that SAWP participants are at least 18 years of age, have agricultural experience and a minimum third grade education, and undergo a medical examination to ensure Canadian health requirements [182]. This suggests that the demographic characteristics of the population of Mexican workers are reasonably uniform. Age ranges of the Mexican workers are reported by the Consulate General of Mexico as being 22-45 years [182]; however the study population in Niagara revealed an age range of 22-65 years (mean 38 years). On average, the number of years of schooling among Mexican workers in Canada has been found to be 7.7 years, which is equivalent to the national mean of Mexico [184]. All but 12.6% of the participants in the present study met the minimum education requirement.

In Canada the vast majority of SAWP workers are men, with women making up only 3% of this population [184], but in the present study women comprised 21.8% of the sample of SAWP workers in the Niagara Region. The proportion of female workers in Niagara has not been previously reported in publications.

Currently, the SAWP includes workers from each of the 32 Mexican states; however the present study population includes participants from 18 centrally located states. This is consistent with the literature reporting that up to 70% of the SAWP participants reside in the six central states, probably due to the fact that recruitment and promotional activities are centered in Mexico City [184].

Generally, the SAWP workers begin arriving in January, and can remain in Canada for a maximum of eight months [182]. At the time of blood sample collection, the study participants had been in Canada for a range of 2-6 months, arriving between February and June. Almost half of the individuals arrived in March, with only 9.3% arriving in June. This differs somewhat from the majority of Mexican SAWP workers, most of whom arrive during the summer months [182]. The average work period each year for the SAWP workers is about 4.9 months, with approximately 73% returning to work in Canada from season-to-season [184]. Overall, the group of Mexican agricultural workers involved in the present study can be considered representative of the population of Mexican migrant workers who come to Canada as part of the SAWP.

5.2 Vitamin D Status of the Study Population

Using a ^{125}I radioimmunoassay (RIA), the overall 25(OH)D concentration of the study population was determined to be 74.9 nmol/L, with concentration means of 68.8 nmol/L and 76.6 nmol/L in females and males, respectively. The prevalences of vitamin D insufficiency and sufficiency were 70.1% and 29.9%, respectively. Global 25(OH)D concentrations of healthy adults are generally considered to be insufficient. A meta-analysis carried out by Hagenau and colleagues [174] reported an overall mean of 54

nmol/L (HPLC and RIA), although studies from African and South American countries were less available than from North America and Europe.

Since regional characteristics have a fundamental impact on determining vitamin D status, it is important to evaluate the vitamin D status of Canadians. Baseline circulating 25(OH)D concentration of a representative sample of healthy Canadian citizens, to date, has not been established. Currently, results from the Canadian Health Measures Survey (CHMS) (2007-2009), a survey that measures health indicators and physical activity levels in a representative sample of Canadians, are being aggregated in order to provide a better understanding of the national prevalence of vitamin D deficiency [185, 186]. The availability of this data will fill an important gap because there are a limited number of studies attempting to establish the vitamin D status of healthy adult Canadians. Vieth and colleagues [187] reported that healthy adults (not consuming vitamin D supplements) working indoors in Toronto (43°N latitude) had winter and summer 25(OH)D concentrations (RIA) of 40.7 and 46.7 nmol/L, respectively. Similarly, Rucker and colleagues [34] evaluated the 25(OH)D concentrations (RIA) of healthy adults (not consuming vitamin D supplements) in Calgary (51°N latitude). Average 25(OH)D concentration was 57 nmol/L during winter, and 72 nmol/L during summer months when UVB exposure was at its peak. These studies highlight the effect of latitude and season on cutaneous vitamin D production in Canadians, which is likely reflected in the vitamin D status of Mexican workers as well.

Regions above 42°N latitude have no UVB exposure during winter months, and therefore no cutaneous production of vitamin D at this time. The latitude of the central

Mexican states from which most of the study participants originated is approximately 20°N [105]. Therefore, workers may have lower 25(OH)D concentrations while residing in Niagara than while in Mexico. Acknowledging that the long hours working outdoors may compensate for the strength of the Mexican sun, effect of long hours are likely minimized by the workers' apparel of long sleeves, pants and hats [188]. Currently, there is no published data reporting the circulating 25(OH)D concentrations of Mexican agricultural workers in Canada, nor those of healthy adults living in Mexico [189-191].

Two studies carried out among post-menopausal women in Mexico found average 25(OH)D concentrations of approximately 65 nmol/L, 67% with insufficient concentrations (<75 nmol/L) (RIA and competitive protein binding assay) [105, 192]. Although this study population is not comparable to the present study, it is interesting to note that despite the differing age groups these concentrations are similar to those of women in the present study. This may be attributed to Mexico's latitude and resulting increase in UVB exposure. Therefore adult Mexicans may likely be vitamin D replete and have fairly homogeneous circulating 25(OH)D concentrations [189]. In the present study, serum 25(OH)D concentration did not significantly differ between age categories, nor did prevalence of vitamin D insufficiency have a statistically significant relationship with age category. This trend has been previously reported [170, 193, 194], although some studies report a decreasing 25(OH)D concentration with increasing age [92, 195] as expected due to reduced cutaneous vitamin D_3 production with increasing age [53].

Recently, access to unpublished serum 25(OH)D RIA results from 46 Mexican family members of SAWP workers from Puebla were found to have a mean circulating

25(OH)D concentration of 68.1nmol/L (65.6 nmol/L in females and 76.9 nmol/L in males), 78% with insufficient vitamin D status. The overall mean 25(OH)D concentration was likely much lower due to the larger proportion of female participants (78%), compared to the present study (21.8%) (Pezzat et al, unpublished data). The present study did not find significantly different 25(OH)D concentration between males and females, which is supported by some literature (Vecino-Vecino 2005; Kudlacek 2003), although others have found significantly higher concentrations in males than females (Scragg 2008; Skull 2008). These differences are usually associated with religious affiliation that requires women to be fully covered. There is no biologically identified explanation for differences between males and females, which are likely related to lifestyle or occupational characteristics.

5.3 Predicting 25(OH)D Concentrations

According to various studies, there are multiple factors that can be used to predict vitamin D status, including age [173], skin pigmentation [174], sun exposure [49], diet [175], latitude [174], alcohol consumption [177] and season [167]. For immigrants to higher latitudes, skin pigmentation [152, 153], latitude [153], length of time in the host country [196] and season [176] play important roles in determining vitamin D status.

In the study, females were more likely to have lower 25(OH)D concentrations than males. The effect of sex on prediction of 25(OH)D concentration has been previously described in immigrant populations. Skull and colleagues [196] aimed to establish the prevalence and risk factors for vitamin D deficiency among immigrants from East Africa, finding that women had higher odds of deficiency than males. As most of these

participants were of the Muslim religion, this may have been caused by a lack of sun exposure due to protective clothing. In the present study, this relationship between men and women may be due to the types of jobs given to men and women, as women generally undertook indoor jobs, such packing [188]. Factors such as the younger age of women and fewer numbers of months in Canada were controlled for in this model.

As expected, an increased number of years involved in the SAWP, as a proxy for age, was a significant predictor of reduced 25(OH)D concentration. As previously mentioned, increased age has been associated with a reduced capacity to produce 7-DHC in the epidermis, thereby reducing the amount of vitamin D produced in response to UVB exposure [53]. The age of participants was correlated with the number of years involved in the program, as older age indicates an increased opportunity to return. This result was mirrored in the logistic regression, which showed a greater likelihood of insufficiency with each year of returned enrolment and therefore each year increase in age. Rucker [34] found that increased age was significantly associated with lower 25(OH)D concentrations, regardless of season. Looker [167] found the same pattern for 25(OH)D concentration, however the effect of age was weaker during the winter months than summer.

In the present study, a longer stay in Canada predicted lower 25(OH)D concentrations and insufficiency. This trend was likely due to two factors. Firstly, the length of time spent at higher latitude than Mexico, and therefore at a reduced UVB exposure, may have reduced vitamin D production in the skin. This relationship has been previously observed in immigrant populations. Skull [196] observed in a cross-sectional study that an increased number of months in Australia predicted vitamin D deficiency

among East African immigrants, with the likelihood of vitamin D deficiency increasing fivefold after two years. There is a lag of approximately two months before an observed reduction of circulating 25(OH)D, due to its slow mobilization (along with vitamin D) from adipose tissues, which act as storage sites [92]. Secondly, since samples were taken in summer, those who have been longer in Canada may have experienced some time where UVB exposure was not possible, while those who spent fewer months in Canada arrived later, and therefore did not experience a period without UVB. As previously mentioned, at 42°N latitude UVB does not reach the Earth's surface between November and February [34], yielding an effect of seasonality on vitamin D status. This effect of seasonality was recently described by Moreno-Reyes [193], who found the peak 25(OH)D concentration of immigrants to Brussels during July-September to be significantly higher than the concentration measured in January-March or April-June, indicating that during winter months there is limited UVB exposure.

5.4 Vitamin D and LTBI Status

Since majority of TB cases in developed regions are attributed to reactivation of LTBI, factors associated with immigration are thought to play a role [122]. A severe vitamin D deficiency (<10 nmol/L [152] and <25 nmol/L [8]) has previously been associated with high prevalence of active TB among foreign-born individuals living at higher latitudes. Establishing the vitamin D status of individuals with and without LTBI may provide insight into immune status or potential for reactivation. The present study did not find a significant difference between the serum 25(OH)D concentrations of LTBI positive (74.1 nmol/L) and negative individuals (75.1 nmol/L), and similarly no

relationship between vitamin D status and LTBI status was found. However, it should be noted that the present study did not have sufficient power, and a larger sample size would provide a more accurate description of these relationships. The relationships between either vitamin D status or 25(OH)D concentration and LTBI status have not been well described in the literature.

A handful of retrospective studies have attempted to investigate the relationship between these factors. Two studies did not find differing 25(OH)D concentrations by LTBI status, while one identified a significant difference. Firstly, a UK clinic found 86% vitamin D insufficiency (<75nmol/L, LC-MS/MS) among children with TB and LTBI, with 79% insufficiency identified in LTBI positive children alone. TB/LTBI status did not have a significant effect on the vitamin D status of these children [10]; however, LTBI diagnostic methods were not identified and there was no control group (LTBI negative participants) with which to compare 25(OH)D concentrations. Secondly, vitamin D status was compared between TB patients and healthy controls in West Africa, with healthy controls including LTBI positive and negative individuals (TST). Similarly to the present study, vitamin D deficiency (LC-MS/MS) was not significantly different between LTBI positive and negative groups [9]. However, the low specificity and large number of false positives of the TST may have yielded inaccurate results. Lastly, differences in serum 25(OH)D concentrations between LTBI positive and negative individuals (IGRA) were shown among African immigrants in Australia [8]. This final study did not specify the type or consistency of vitamin D assay. All included records were taken from one hospital, indicating that the same vitamin D assay was utilized, though these findings must be interpreted with caution. These studies provide insight into the lack of

consistency and accuracy in determining the potential role of vitamin D in LTBI, as well as highlight the importance of studying this relationship in immigrant populations. Differences in these samples would need to be followed longitudinally to determine if low 25(OH)D concentrations resulted in reactivation.

While still considered insufficient, participants in the present study were not severely vitamin D deficient. This may be explained by the fact that the participants worked long hours outdoors during summer months when samples were collected. However, the previously mentioned work attire of long sleeves, pants and hats for avoidance of scratches and chemicals [188], likely also prevented large increases in cutaneous vitamin D production. Immigrants to non-endemic TB regions normally experience a wintertime reduction in vitamin D production, which is associated with a subsequent late spring or early summer increase in TB diagnosis due to LTBI reactivation [149, 151]. The workers' continuous return to Mexico at summer's end provides them with UVB exposure unavailable during Canadian winters, thereby avoiding long periods without cutaneous vitamin D production.

Vitamin D metabolites are known immune regulators that inhibit the Th1 pro-inflammatory reactions of the adaptive immune system, some of which are important for preventing LTBI reactivation by maintaining the integrity of the granuloma. This may seem paradoxical, but the down-regulation of circulating pro-inflammatory cytokines likely prevents potentially detrimental effects to lung or other tissues. The granuloma provides the host with a physical barrier within which inflammatory cytokines are concentrated. In this setting, pro-inflammatory cells such as T cells and macrophages are

brought into close proximity with *M. tuberculosis* [124], allowing for intracellular bacterial killing by anti-mycobacterial cathelicidin. With an increase from 23 nmol/L to 78 nmol/L, Liu and colleagues [14] observed a 2.5 fold increase in cathelicidin mRNA production in human monocytes cultured in human serum. As well, this reaction was dependent on the presence of a TLR2/1 ligand, such *M. tuberculosis*. Therefore circulating 25(OH)D may ensure local production of $1,25(OH)_2D$, and therefore cathelicidin, in macrophages infected by *M. tuberculosis* in the granuloma during LTBI.

The local production of the active metabolite $1,25(OH)_2D$ in immune cells has also been observed by Lin and colleagues [197] in non-human primates. In cynomolgus macaques, calcification of tuberculous granulomas indicated the local production of $1,25(OH)_2D$, suggesting that the $1,25(OH)_2D$ concentration inside the granuloma differs from the $1,25(OH)_2D$ production in other tissues. Therefore Th1 depression may only be enough to prevent over-production of $1,25(OH)_2D$ that would lead to hypercalcemia and potential toxicity outside of the granuloma. This may partly explain the results of the present study showing no significant difference between circulating 25(OH)D concentrations of LTBI positive and negative participants, as each of these groups are clinically healthy, indicating that vitamin D metabolite concentrations are maintained in balance with immune reactions.

5.5 25(OH)D and IFN-γ Concentrations

Among LTBI positive individuals, there was a moderate, positive correlation between serum 25(OH)D concentrations and IFN-γ concentrations (released by T cells in response to *M. tuberculosis* antigens). This correlation did not exist when all participants

were included (i.e., LTBI positives and negatives), because LTBI negative individuals lacked previous exposure to *M. tuberculosis* antigens required for the stimulated release of IFN-γ from peripheral effector T cells. There are various mechanisms by which vitamin D metabolites interact with the immune system, some of which are better understood than others.

A precise interpretation of differing *M. tuberculosis*-stimulated IFN-γ production has yet to be established. Recent *M. tuberculosis* infections generally result in a greater secretion of IFN-γ. Effector cells that have been recently exposed to *M. tuberculosis* antigens *in vivo* are likely to respond to re-exposure with rapid IFN-γ release [198]. Therefore, workers presenting with higher IFN-γ concentrations may have been more recently exposed. This could be due to antigenic stimulus by primary infection, re-exposure while in Mexico or granulomatous breakdown that was later contained. As well, higher IFN-γ concentration may indicate incipient active TB among recently infected individuals [199]. Therefore, if any of these individuals were recently infected, this could suggest future reactivation. This same relationship has not been reported in individuals known to have a long time latent infection.

The immune mechanisms involved in this relationship are not yet fully understood. $1,25(OH)_2D$ down-regulates transcription and secretion of Th1cytokines IFN-γ, IL-12 and TNF-α in *M. tuberculosis*-infected macrophages, DCs, T cells and PBMC in culture [102, 157]. As previously stated, Th1 cytokines are important for maintaining the granuloma, and therefore preventing reactivation [200, 201]. This down-regulation of IFN-γ by $1,25(OH)_2D$ seems to contradict the findings of the present study and the established

association between vitamin D deficiency and active TB. However, 25(OH)D is the inactive precursor of $1,25(OH)_2D$, and is not an indicator of $1,25(OH)_2D$ activity.

This positive correlation may be indicative of a chance relationship between circulating 25(OH)D concentrations and IFN-γ release by effector T cells. Firstly, there was not sufficient power in this Spearman correlation to accept its outcome. Secondly, upon further consideration it was concluded that carrying out an analysis of these two variables had numerous weaknesses. For example, the *in vitro* nature of the IFN-γ release assay meant that a large amount of TB antigen was used to induce a hyper-response from the effector T cells. Under natural conditions, effector T cells would not be exposed to such a high concentration of TB antigens. As well, the presence of TB antigen may have overridden any down-regulation of IFN-γ by $1,25(OH)_2D$. Lastly, it must be acknowledged that two different samples were utilized to determine the IFN-γ and 25(OH)D concentrations. The 25(OH)D concentrations were measured directly from serum samples, while the IFN-γ release was measured following stimulation of T cells in whole blood. It is possible that circulating 25(OH)D or $1,25(OH)_2D$ could have been affected by the sample manipulation during the IGRA. Since 25(OH)D concentration was not measured following completion of the IGRA, this cannot be disregarded.

Overall, the average 25(OH)D concentration of this population, while below the determined cut-point of sufficiency does not indicate severe deficiency. As none of the workers showed signs of LTBI reactivation, concentrations of 25(OH)D slightly lower than optimal may still influence immunological protection in the management of LTBI. This study highlights the need to gain a better understanding of vitamin D status of

migrant workers as it relates to LTBI reactivation, and underscores the importance of nutritional health promotion in this population.

5.6 Study Strengths

The ^{125}I RIA (DiaSorin Inc., Stillwater, MN) utilized in the study is the most commonly used assay for quantifying circulating 25(OH)D concentrations in human serum. It is the assay for which other assays are calibrated, and its use allows for the comparison of 25(OH)D concentrations and vitamin D insufficiency prevalence values with many published studies. It provides results in a relatively short amount of time and for a larger number of serum samples. The 25(OH)D RIA also has 100% cross-reactivity between the $25(OH)D_2$ and $25(OH)D_3$ vitamin D metabolites, and therefore provides a more accurate measurement of the total circulating 25(OH)D than some other assays.

Determination of LTBI status (by Duarte) was carried out with an IGRA (QuantiFERON Inc., Cellestis Ltd., Australia) and used as a confirmatory test for TST results. The TST required by the Public Health Agency of Canada is not always indicative of a current TB infection, as it is affected by Bacille Calmette-Guérin (BCG) vaccination and other environmental mycobacteria. In absence of a gold standard test for LTBI, the IGRA is the preferred method of determining LTBI positivity, as it is able to distinguish between LTBI and a BCG-vaccinated individual and has higher specificity and sensitivity than the TST.

This was the first study to report the vitamin D status of Mexican migrant workers in Canada's Niagara Region, and results can be generalized to Mexican workers in other

parts of Canada. As well, in the absence of vitamin D data for healthy Mexican adults, results from this study may provide an indication of the vitamin D status of healthy Mexican adults.

In the absence of any other vitamin D data in this population, the cross-sectional design of the study allowed for the relatively fast collection of preliminary data. This provided a snapshot of the prevalence of vitamin D insufficiency and related characteristics, and how they relate to LTBI status.

5.7 Study Limitations

This study made use of the previously completed survey (developed by Duarte and Sanchez) that focused on risk factors for LTBI. Therefore some important predictors of vitamin D status were unavailable for analysis, such as skin pigmentation, diet and nutrition, type of work done by the participants (indoor versus outdoor) and a measurement for sun protection. The inclusion of these variables would have allowed for a more in-depth analysis of predictors of vitamin D insufficiency. However, some of these variables, while important risk factors for insufficiency, may add further limitations to the study. Inclusion of diet and nutrition variables to ascertain dietary intake of vitamin D would have to be very specific. Dietary intake relies on participants' ability to recall past actions in detail, and may be difficult to analyze due to differing fortification systems and vitamin D content of fish. As well, the type of work and sun protection behaviours may change from day to day and so would have to be carefully evaluated.

This was a convenience sample, and therefore not randomly selected. Potential participants could only be approached among farms agreeing to participate and within a limited timeframe at the end of the summer season. While convenience sampling tends to reduce the generalizability of results, the nature of some of the characteristics being measured in this study, such as LTBI status and serum 25(OH)D concentrations, may not be strongly influenced by this limitation. In addition, the small sample size of the study limited the number of individuals that could be analyzed for LTBI positivity or vitamin D deficiency.

5.8 Future Research

5.8.1 Conducting Longitudinal Studies

In order to provide an indication of the potential role of vitamin D metabolites in LTBI reactivation, a longitudinal study must be carried out in which the 25(OH)D concentrations of LTBI positive individuals (and uninfected controls) are monitored over a span of at least five years. 25(OH)D concentrations would be evaluated seasonally. This would likely be carried out among immigrants to Canada from TB-endemic regions, as reactivation is likely to occur within the first five years of arriving in the host country, and active TB is likely due to reactivation of latent infection. This would allow for a more accurate association between LTBI status and vitamin D status, as well as the potential of vitamin D deficiency (and severity of deficiency) to influence reactivation of latent infection. This knowledge may be beneficial to public health agencies monitoring and perhaps predicting the levels of reactivation in Canada.

5.8.2 Assessment of Fluctuation of Cytokine Profile in LTBI Positive Individuals

The cytokine profile of LTBI positive individuals, including concentrations of Th1 cytokines TNF-α and IFN-γ, and how they fluctuate have not yet been examined. Further analysis of these cytokines, important to the maintenance of the granuloma during latent infection, may help to evaluate if there is a shift in immune balance over time and in relation to vitamin D status, especially 25(OH)D concentrations. While $1,25(OH)_2D$ has been shown to inhibit the production of these cytokines, there is currently no known association between concentrations of these cytokines and 25(OH)D, the metabolite

measured in the determination of vitamin D status and the measure of exposure and supply of the nutrient to target tissues. This may suggest or lead to some other as yet undefined immune interactions in place.

5.9 Conclusions

This study added to two important and growing bodies of literature. Firstly, this study provided circulating 25(OH)D concentrations of healthy adult Mexican men and women living in Canada, which have not been reported before. Secondly, publications focusing on the relationship between LTBI status and vitamin D insufficiency are few. This is the first time this relationship was evaluated in a group of migrant workers from a TB-endemic sub-tropical region temporary employed and residing in a non-endemic region.

The majority of Mexican migrant workers were vitamin D insufficient, but very few were severely deficient, indicating the vitamin D status may not have been greatly reduced during the temporary stay in Canada. This was likely due to participants' return to the lower latitudes of Mexico following Niagara's growing season, and their subsequent avoidance of Canadian vitamin D winters.

No significant difference in vitamin D status between LTBI positive and negative individuals, and the lack of LTBI reactivation, indicated that although the 25(OH)D concentrations were lower than optimal, they were likely high enough to maintain immunological protection against LTBI reactivation. Determining if a critical 25(OH)D concentration for reactivation exists remains a challenge and a potential focus of future prospective studies.

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APPENDIX I: Ethics Approval

Brock University Research Ethics Board (REB)

Request for Ethics Clearance of a **Revision or Modification** to an Ongoing Application to Conduct Research with Human Participants

Please refer to Brock University Research Ethics Guidelines, found at http://www.brocku.ca/researchservices prior to completion and submission of this application. If you have questions about or require assistance with the completion of this form, please contact the Research Ethics Office at (905) 688-5550 ext. 3035, or reb@brocku.ca. Once complete, please return this form with all accompanying material to **MacKenzie Chown D250A.**

SECTION A – GENERAL INFORMATION

1. **Title of the Research Project:** Preliminary epidemiological study of tuberculosis in Mexican agricultural workers in the Niagara Region, Canada.

2. **Principal Investigator:** Angela Duarte — **File Number: DUARTE** 06-288

   Department: Community Health Sciences — **Email:** angela.duarte@brocku.ca

3. **Faculty Supervisor:** Dr. Ana Sanchez — **Department:** Community Health Sciences

   Email: ana.sanchez@brocku.ca — **Phone Ext.:** 4388

4. **Original Approval Date:** March 15, 2007 — **Anticipated Closing Date:** December 30, 2008

SECTION B – MODIFICATIONS/REVISIONS

5. Provide a brief description of, and explanation for, the revision(s) or modification(s) requested to your application that previously received ethics clearance. If the revision(s) is (are) to a questionnaire or interview protocol with previous ethics clearance, submit the entire document and **highlight the sections that are revised or added**. A complete copy of any **new** measure(s) or scale(s) must be attached for ethics review.

We would like to include Dr. Paul LeBlanc from the Department of Community Health Sciences as a co-investigator so that the proposed vitamin D analyses can be performed. Dr. LeBlanc was awarded a Brock University Advancement Fund to examine the levels of pro-vitamin D and their association with cellular immunity against tuberculosis in a sample of healthy Mexican agriculture workers in the Niagara Region. This modification to the previously accepted REB will allow for a release of funds and for the research to proceed.

We would also like to include Timna Merion as a MSc graduate student whose research project will include the examination of vitamin D levels in the aforementioned sample. Timna will learn the lab techniques and perform the assays under Dr Leblanc's and Sanchez supervision.

6. a) Do the revised procedures outlined above require and change(s) to the Informed Letter/Consent Form currently in use and that previously received ethics clearance? [ ] **Yes** [X] **No**

b) If yes, briefly describe these changes. Attach a copy of the revised Information Letter/Consent Form and **highlight the revised sections**.

SECTION C – SIGNATURES

I/We respectfully request ethics approval of the modifications/revisions described above. All modified Documents and procedures have been submitted for REB review and approval.

Signature of Principal Investigator **Date**

Signature of Faculty Supervisor **Date**

***Office Use Only**

The Modification request as described above to an ongoing project involving human participants has been reviewed and received ethics clearance.

Chair, Research Ethics Board **Date**

APPENDIX II: QuantiFERON-TB Gold In-Tube Procedure

Background and Principle of the Assay

During *M. tuberculosis* infection, mycobacterial antigens will trigger the response of cell-mediated immunity (CMI), including the release of the IFN-γ cytokine. This test incorporates proteins (ESAT-6, CFP-10 and TB7.7) that simulate mycobacterial peptides. Individuals who are infected with *M. tuberculosis* have T lymphocytes in their blood that will react to these proteins by releasing IFN-γ, which is then quantified. The antigens used in this test are not present in any strain of BCG, and therefore is not likely to produce a reaction in individuals who have been immunized, but are uninfected. This test is therefore an indicator of LTBI and a supporting test for the diagnosis of active TB. In absence of active TB, positive results of this test could indicate a LTBI. However, other Mycobacteria (ie. *M. kansasii*, *M. szulgai* and *M. marinum*) may also yield positive test results [172].

Whole blood is collected directly into three specialized tubes, including a Nil Control tube, TB Antigen tube (containing the protein cocktail), and a Mitogen Control tube that controls for proper blood processing techniques and unknown immune status of participants. For example, a low IFN-γ response (<0.5 IU/mL) in the Mitogen tube and negative response in the TB Antigen tube indicates an indeterminate response. A TB Antigen tube is considered to have a positive IFN-γ response when it is significantly higher than the IFN-γ response of the Nil tube. The IFN-γ response of the Nil tube is

subtracted from that of the TB Antigen and Mitogen tubes, in order to adjust for non-specific IFN-γ present in the blood [172].

Assay Technique

Stage One: Blood sample collection and storage (This stage was completed in 2007as a component of Angela Duarte's Master's thesis project). Using 70% ethanol, a certified phlebotomist disinfected the arm of the participant from which the blood sample was taken. A tourniquet was applied 3-4 inches above the puncture site, and 5 mL of blood extracted from the larger median cubital, basilica or cephalic veins into a vacutainer tube without anticoagulant. Samples were stored and transported to Brock University in an ice box. At the laboratory in Brock University, the samples were centrifuged and stored at -20°C until they were used.

Stage Two: Human IFN-γ ELISA. Before use, plasma samples and reagents were equilibrated to room temperature (22°C ± 5°C) for approximately 60 minutes. Strips that were not utilized were removed from the microplate frame, and stored in the refrigerator in a sealed foil pouch. The freeze dried Kit Standard was reconstituted using 0.25 mL distilled water (as indicated on the Standard vial), and gently mixed. A 1 in 4 dilution series of IFN-γ in Green Diluent was produced using the reconstituted Kit Standard. Standards were assayed in duplicates. Two sets of four tubes will be labelled: "S1", "S2", "S3" and "S4", to each of which 150 µL of Green Diluent was added. 150 µL of the Kit Standard was added to the tubes labelled "S1", and thoroughly mixed. 50 µL of the contents of "S1" were then transferred to "S2", and thoroughly mixed. 50 µL of the

contents of "S2" were be transferred to "S3", and thoroughly mixed. "S4" contained only Green Diluent, which was the zero standard. The concentrations of the standards "S1", "S2", "S3" and "S4" were then be 4.0, 1.0, 0.25 and 0 IU/mL, respectively.

Freeze dried Conjugate 100X was reconstituted with 0.3 mL of distilled water, and gently mixed. Working Strength Conjugate was produced by diluting the reconstituted Conjugate 100X Concentrate in Green Diluent, according to Table 1, and mixed gently.

Table 1: Conjugate Preparation [172]

NUMBER OF STRIPS	VOLUME OF CONJUGATE 100X CONCENTRATE	VOLUME OF GREEN DILUENT
2	10µL	1.0mL
3	15µL	1.5mL
4	20µL	2.0mL
5	25µL	2.5mL
6	30µL	3.0mL
7	35µL	3.5mL
8	40µL	4.0mL
9	45µL	4.5mL
10	50µL	5.0mL
11	55µL	5.5mL
12	60µL	6.0mL

Plasma samples were vortexed to ensure the even distribution of IFN-γ in each sample. A multichannel pipette was used to transfer 50 µL of Working Strength conjugate to the ELISA microplate wells, and then 50 µL of the plasma samples was added to the appropriate wells of the plate. Lastly, 50 µL of each of the standards was added to the appropriate wells. The plate was thoroughly mixed on a microplate shaker for one minute,

and then covered with a lid and incubated at room temperature (22°C ± 5°C) for 120 ± 5 minutes (out of direct sunlight).

Working Strength was buffer was prepared by diluting one part Wash Buffer 20X Concentrate with 19 parts distilled water, and thoroughly mixed.

Following incubation, the wells were washed with 400 µL of Working Strength wash buffer for at least six cycles in an automated ELISA plate washer. Once the automated washer had finished, the plates were taken out and excess wash buffer was tapped upside down on Kimwipes. 100 µL of Enzyme Substrate Solution was added to each well and thoroughly mixed with the microplate shaker. The plate was covered with a lid, and incubated at room temperature (22°C ± 5°C) for 30 minutes (out of direct sunlight).

Following incubation, 50 µL of Enzyme Stopping Solution was added to each well and mixed. The Enzyme Stopping Solution was added to the microplate wells following the same order and speed as the substrate.

Within 5 minutes of adding the Enzyme Stopping Solution, the microplate was placed in a reader (with 450 nm and 620-650 nm reference filters) and the optical density was measured. QuantiFERON-TB Gold IT Analysis Software was used to analyze raw data and calculate the results, producing a standard curve.